NACO

HIV TESTING MANUAL

LABORATORY DIAGNOSIS, BIOSAFETY AND QUALITY CONTROL

सत्यमेव जयते

NATIONAL INSTITUTE OF COMMUNICABLE DISEASES
Directorate General of Health Services, Government of India

NATIONAL AIDS CONTROL ORGANISATION
Ministry of Health & Family Welfare, Government of India

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HIV TESTING MANUAL:
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NATIONAL INSTITUTE OF COMMUNICABLE DISEASES

Government of India, Directorate General of Health Services,
22, Shamnath Marg, Delhi-110 054

and

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FOREWORD

The Acquired Immuno Deficiency Syndrome (AIDS) was first recognised in the United States in 1981. The aetiological agent, Human Immuno deficiency Virus (HIV), was isolated in 1984 and laboratory test for HIV became commercially available, thereafter. This enabled health care workers to diagnose HIV infection, individuals to know whether or not they were infected, and most importantly medical science to understand HIV/AIDS better. The availability of these tests has also led to misconceptions regarding the role of HIV testing.

Compared to any other type of disease, the issues related to the diagnosis of HIV infection are far more complex. AIDS is invariably fatal and infection is lifelong. No drugs are available to cure AIDS or to render an HIV infected person, non-infectious. Since HIV is spread mainly through sexual contact, individual known to be infected with HIV are unfortunately often stigmatised and discriminated against. Such a situation results from the lack of proper understanding regarding the mode of transmission. Many individuals, particularly health care workers, seem to believe that the spread of HIV can be controlled by identifying people with HIV infection, and testing of hospital patients is often advocated. This is mainly due to ignorance about the objectives of HIV testing. A manual on HIV testing will be helpful in imparting adequate training to medical fraternity and para medical workers.

The laboratory plays an important role in the National AIDS Control Programme. It can assist programme managers in studying the trend of epidemic in the community; ensures safe blood, free from HIV infection, and may assist physicians to assess the prognosis of the infection. Apart from this, it can assist the individual to modify his behaviour or life style to prevent the spread of HIV in the community and to improve the quality of his life.

To facilitate the training of laboratory staff in HIV Testing Procedures, a reference manual was very much needed to improve the skill of laboratory staff and other functionaries involved in HIV/AIDS prevention and control programme.

It is expected that the present manual which was prepared under the supervision of the National Institute of Communicable Diseases will fulfil this need.

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PREFACE

HIV infection is spreading rapidly in India. The infection can only be detected by laboratory tests, as there is a long asymptomatic period when the individual is infectious and can spread disease but has no specific symptoms or signs of disease. The role of the laboratory is very important and it is essential that the highest standards are not only maintained in each laboratory but are also regularly monitored.

There are a number of diagnostic procedures and various types of commercial diagnostic kits available. Moreover the results of the laboratory tests can be influenced by many factors. It is essential that each laboratory has a Standard Operative Procedure (SOP) which details each step of the test including bio-safety precautions. The laboratories are also expected to follow the national guidelines circulated by NACO on ethical issues related to HIV testing.

The HIV Testing Manual has been prepared as a practical guide for laboratory personnel. The Manual is comprehensive and covers relevant topics of interest. The chapters have been drafted by national experts. The material has been extensively reviewed at two meetings held at NICD.

Dr. Usha K. Baveja, Consultant and Head HIV/AIDS Division and her colleagues Dr. D. Chattopadhya and Sh. Ramesh Aggarwal deserve credit for taking the initiative for the preparation of Manual and hard work put in by them to give it shape in a record time. The National Institute of Communicable Diseases is also obliged to all the experts who willingly contributed the chapters.

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ACKNOWLEDGEMENTS

The "HIV Testing Manual: Laboratory Diagnosis, Bio-safety and Quality Control" is intended for use by different categories of HIV testing laboratories/centres all over India as a reference and training manual. The manual can be referred to, to prepare standard operative procedures (SOPs) and to practise bio-safety and quality control etc., so that each HIV testing facility may uniformly produce quality results within the limitations of the test.

This manual does two things which have not been done before. First it gives the updated background knowledge on HIV and other transfusion transmitted infections along with techniques. Second, it takes advantage of the expert knowledge of authorities by presenting contributions from nearly 18 contributors from different parts of the country.

I most gratefully acknowledge the technical guidance and support from Dr. S.P. Agarwal, Director General of Health Services, Government of India, Ministry of Health & Family Welfare.

I express my sincere gratitude and thanks to Shri Prasada Rao, Additional Secretary and Project Director, NACO for encouragement, financial support and for writing the foreword for the manual. I am thankful to Director, NICD, Ms. Jotna Sokhey for her encouragement, support and constructive comments during the discussions. I thank Dr. Sudershan Kumari, Regional Advisor, Laboratory Services WHO/SEARO for her expert guidance and for providing the relevant literature.

I am most grateful to all of the technical experts who contributed various chapters in a timely manner. I especially thank Dr. P.L. Joshi, Dr. L. Dar, Dr. R.N. Makroo, Dr. K. Ray, Dr. S. Tripathi for their valuable comments and suggestions during the meetings held to review the rough draft. I wish to thank my colleagues Dr. D. Chattopadhya, Shri R.K. Aggarwal and others for their contribution and help during the two meetings for the review of the manual.

Finally, I express my appreciation and thanks for the secretarial assistance provided by Smt. Usha and Sh. Mukesh Kumar.

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Chapter 1

BASIC EPIDEMIOLOGY AND NATURAL HISTORY OF HIV/AIDS

1.1 INTRODUCTION

Acquired Immunodeficiency Syndrome (AIDS) is caused by Human Immunodeficiency Virus (HIV). It is a serious disorder of the immune system in which the body's normal defences against infection break down, leaving it vulnerable to a host of life threatening infections, including unusual malignancies.

1.2 THE PROBLEM

1.2.1 Global

By the end of 1998, according to estimates from the joint United Nations Programme on HIV/AIDS (UNAIDS) and the World Health Organization (WHO), the number of people living with HIV will have grown to 33.4 million, 10% more than just one year ago. The epidemic has not been overcome anywhere. Virtually every country in the world has seen new infections in 1998 and the epidemic is frankly out of control in many places.

According to UNAIDS/WHO estimates, 11 men, women and children around the world were infected per minute during 1998 (near to 6 million). More than 95% of all HIV-infected people now live in the developing world, which has likewise experienced 95% of all deaths to date from AIDS, largely among young adults who would normally be in their peak productive and reproductive years.

Sub-Saharan Africa is home to 70% of the people who became infected during last year (1998). Africa, the global epicentre, continues to dwarf the rest of the world on the AIDS balance sheet. Since the start of the epidemic, 83% of all AIDS deaths so far have been in this region. At least 95% of all AIDS orphans have been African. While no country in Africa has escaped the virus, some are far more severely affected than others. The Southern part of the African continent holds the majority of the world's hard hit countries. In Botswana, Namibia, Swaziland and Zimbabwe, estimates show that between 20-26% of people aged 15-49 are living with HIV or AIDS. HIV prevalence in pregnant mothers was found to be over 20% in majority of sentinel sites in these country.

Increasingly the spotlight is on the spread of HIV through the Asian continent, especially in South Asia and East Asia, while rates remain low relative to some other regions, well over 7 million Asians are already infected and HIV is clearly beginning to spread in earnest through the vast populations of India and China.

In Eastern Europe and in Latin America and the Caribbean, infections are concentrated in marginalized groups though, clearly not limited to them. Some studies suggest that up to 30% of men who have sex with men may be infected; among drug injectors in Argentina and Brazil the proportion may be close to half of above.

In North America and Western Europe, new combinations of anti-HIV drugs continue to reduce AIDS deaths significantly. However, because new infections continue to occur

while antiretroviral drug cocktails keep already infected people alive, the proportion of the population living with HIV has actually grown. **Table 1** shows the regional HIV/AIDS statistic and features.

Table 1. Regional HIV/AIDS statistics and features, December 1998

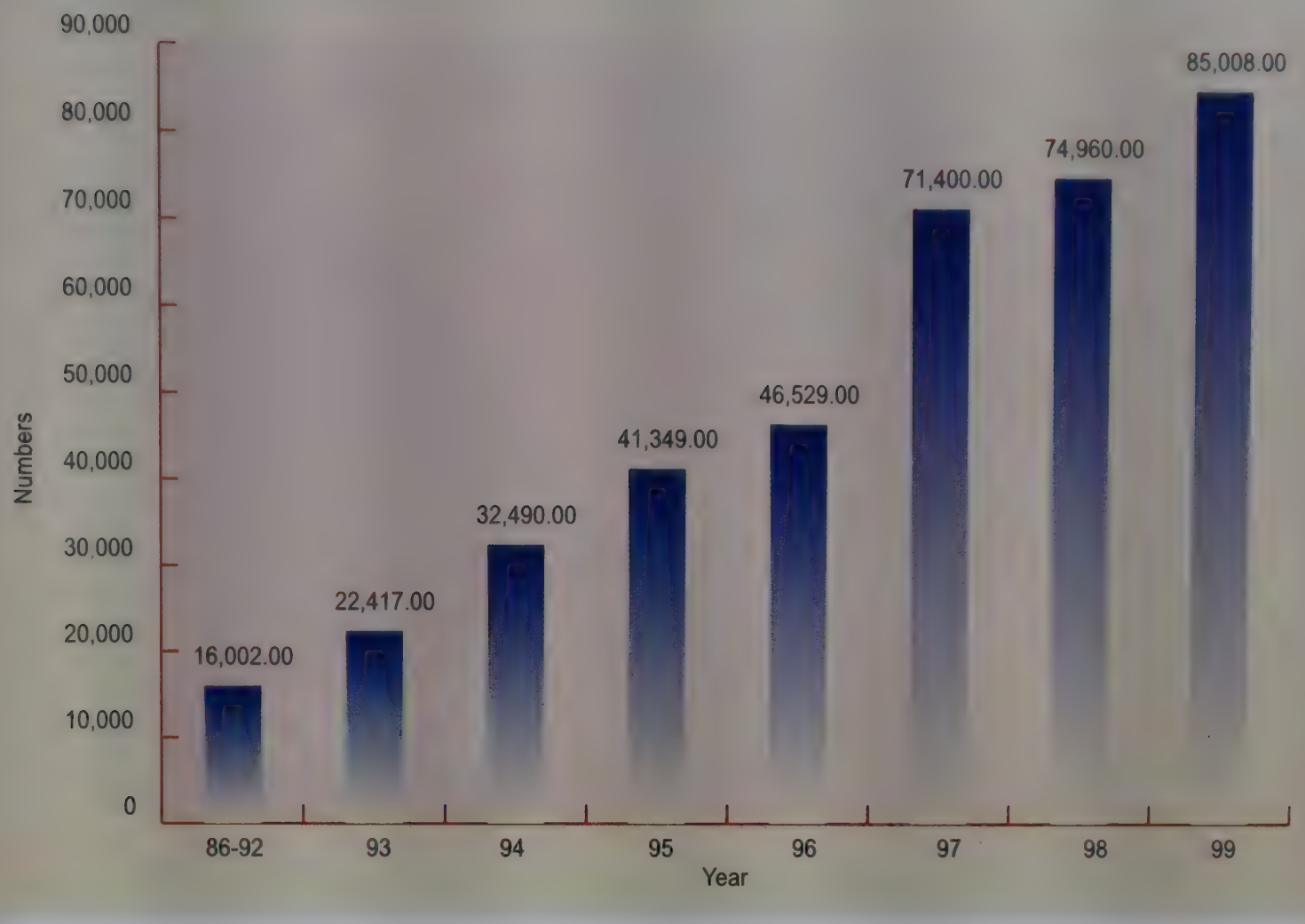
Region	Epidemic started	Adults & children living with HIV/AIDS	Adults & children newly infected (**) with HIV	Adults prevalence rate (*)	per cent of HIV-positive adults who are women	Main mode(s) of transmission (#) for adults living with HIV/AIDS
Sub-Saharan Africa	Late '70s-early '80s	22.5 million	4.0 million	8.0%	50%	Hetero
North Africa & Middle East	Late '80s	210 000	19 000	0.13%	20%	IDU, Hetero
South & South-East Asia	Late '80s	6.7 million	1.2 million	0.69%	25%	IDU, Hetero MSM
East Asia & Pacific	Late '80s	560 000	200 000	0.068%	15%	MSM, IDU, Hetero
Latin America	Late '70s-early '80s	1.4 million	160 000	0.57%	20%	Hetero, MSM
Caribbean	Late '70s-early '80s	330 000	45 000	1.96%	35%	Hetero, MSM
Eastern Europe & Central Asia	early '90s	270 000	80 000	0.14%	20%	IDU, MSM
Western Europe	Late '70s-early '80s	500 000	30 000	0.25%	20%	MSM, IDU
North America	Late '70s-early '80s	890 000	44 000	0.56%	20%	MSM, IDU, Hetero
Australia & New Zealand	Late '70s-early '80s	12 000	600	0.1%	5%	MSM, IDU
TOTAL		**33.4 million**	**5.8 million**	**1.1%**	**43%**	

* The proportion of adults (15 to 49 years of age) living with HIV/AIDS in 1998, using 1997 population numbers.
# MSM (sexual transmission among men who have sex with men), IDU (transmission through injecting drug use), Hetero (heterosexual transmission).
UNAIDS/98.35 WHO/EMC/VIR/98-4-WHO/ASD/98.3
** Infections occurred during the year 1998.

1.2.2 India

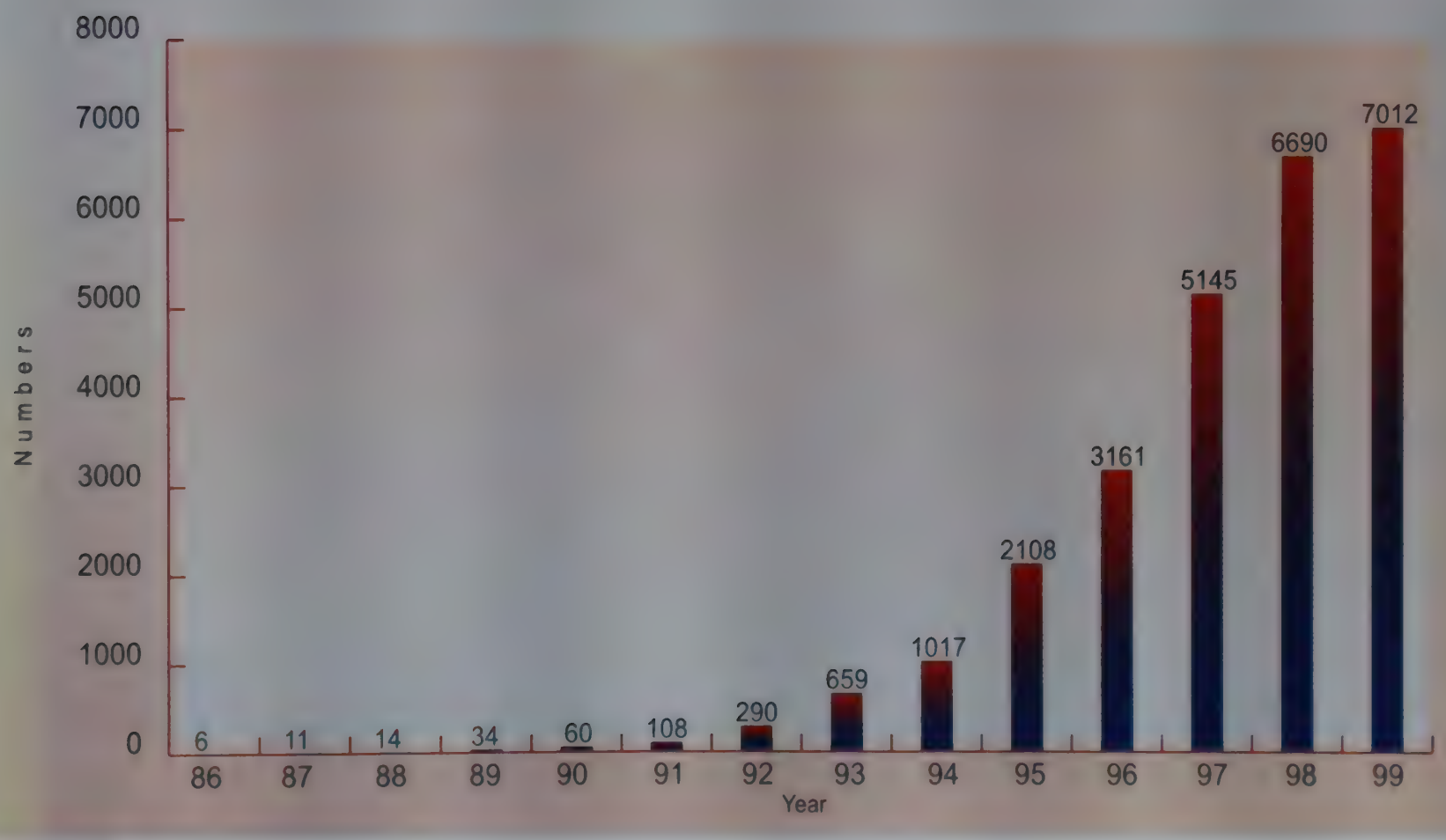
Since the detection of HIV infection in commercial sex workers (C.S.Ws) in Tamil Nadu in 1986, the infection is growing very fast in the country. As on March 1999, 85,008 HIV infected persons and 7012 AIDS case were reported to NACO. Although the magnitude of disease has been found to be varied in various parts of the country, the states of Maharashtra, Tamil Nadu, Andhra Pradesh, Karnataka and Manipur are hard hit states. Maharashtra accounts for close to 50% of all reported HIV and AIDS cases in India. Sentinel surveillance data collected during August-October, 1998 revealed that in the above mentioned States the infection in pregnant mothers, in high risk groups like STD clinic attenders, C.S.Ws, injecting drug users etc. has shown galloping increases. Moreover, the estimated number of HIV infections in the country have been reported to be 3-5 million. **Fig. 1** through **6** show the HIV/AIDS features and statistics in India.

FIG. 1.1 CUMMULATIVE HIV INFECTIONS IN INDIA
(MAY 1986 - MARCH 1999)



Source: NACO

FIG. 1.2 CUMMULATIVE AIDS CASES IN INDIA
(MAY 1986 - MARCH 1999)



Source: NACO

FIG. 1.3 CUMMULATIVE SEROPOSITIVITY RATE OF HIV INFECTION AMONG WOMEN BLOOD DONORS IN INDIA (UPTO MARCH 1999)

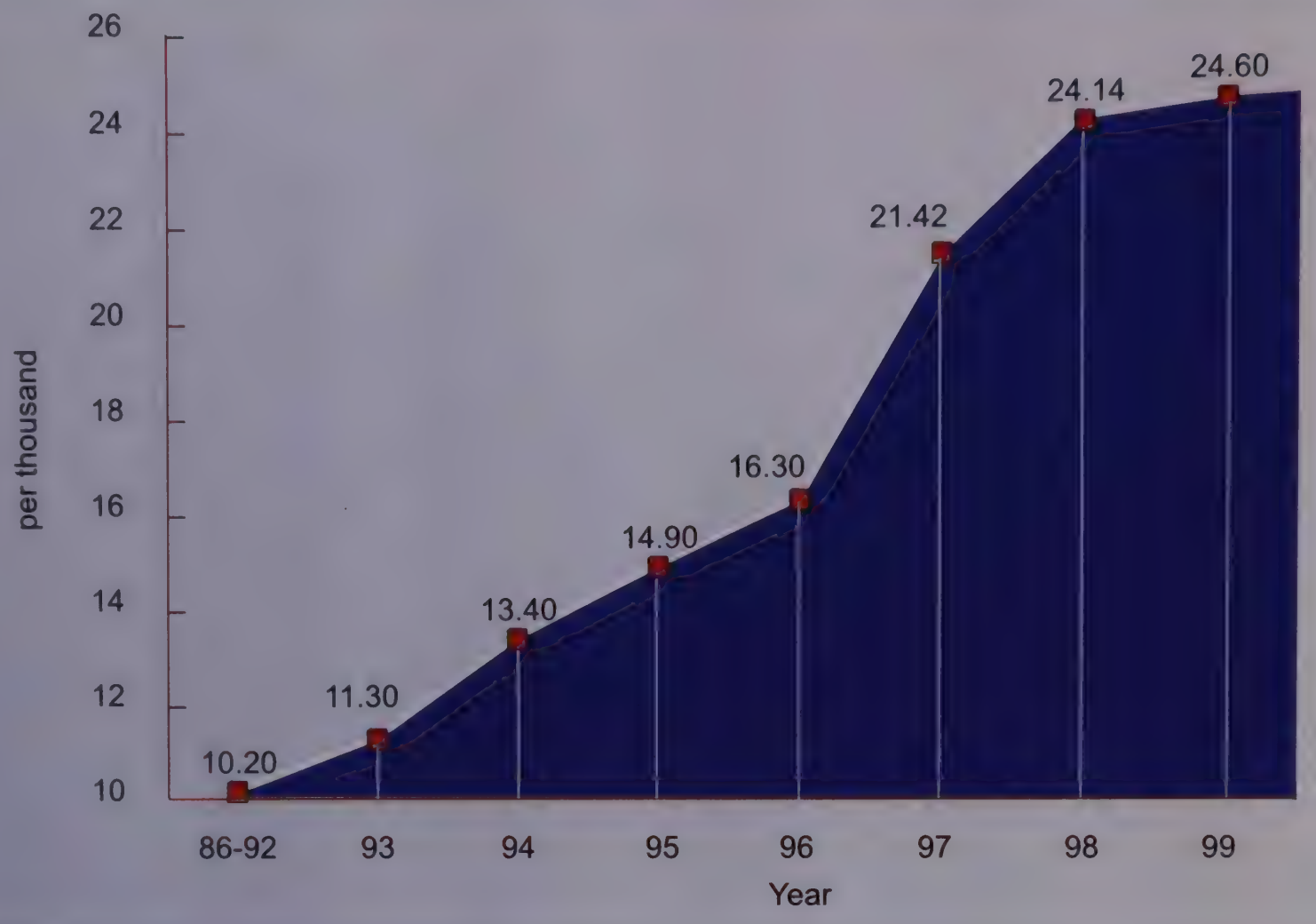
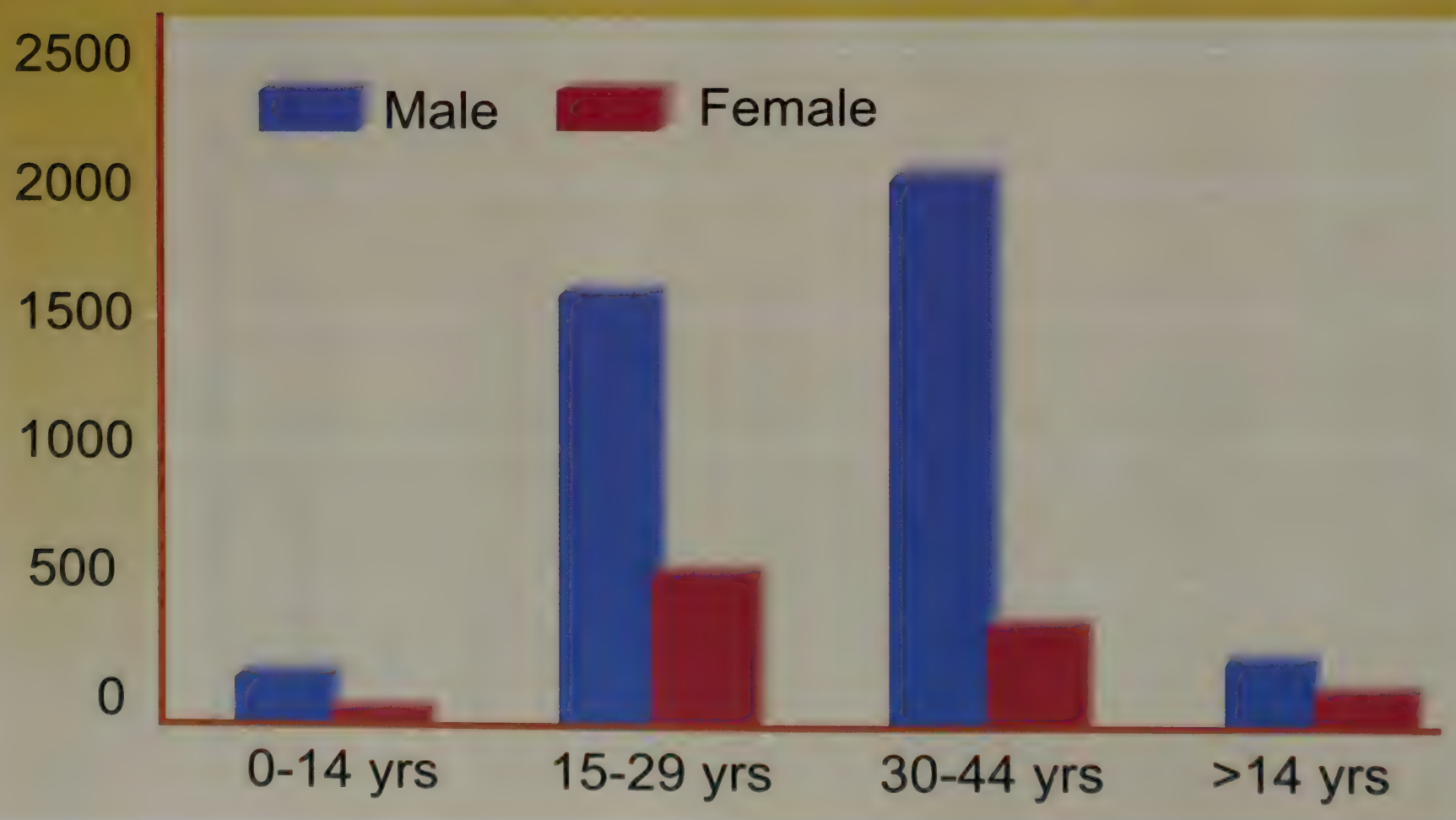
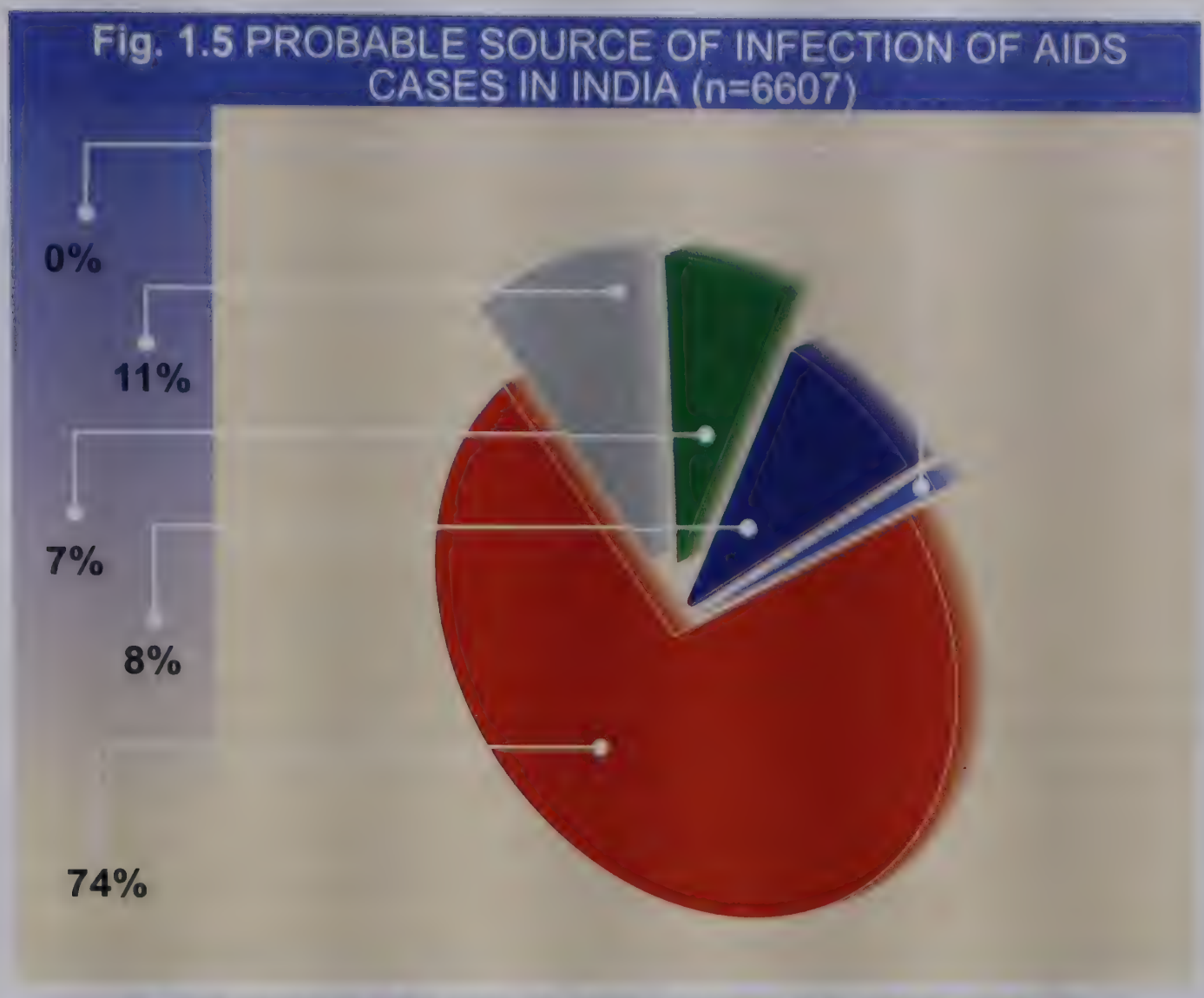


Fig. 1.4 AGE & SEX DISTRIBUTION OF AIDS CASES IN INDIA
(MALES - 78.9%, FEMALES - 21.1 %)





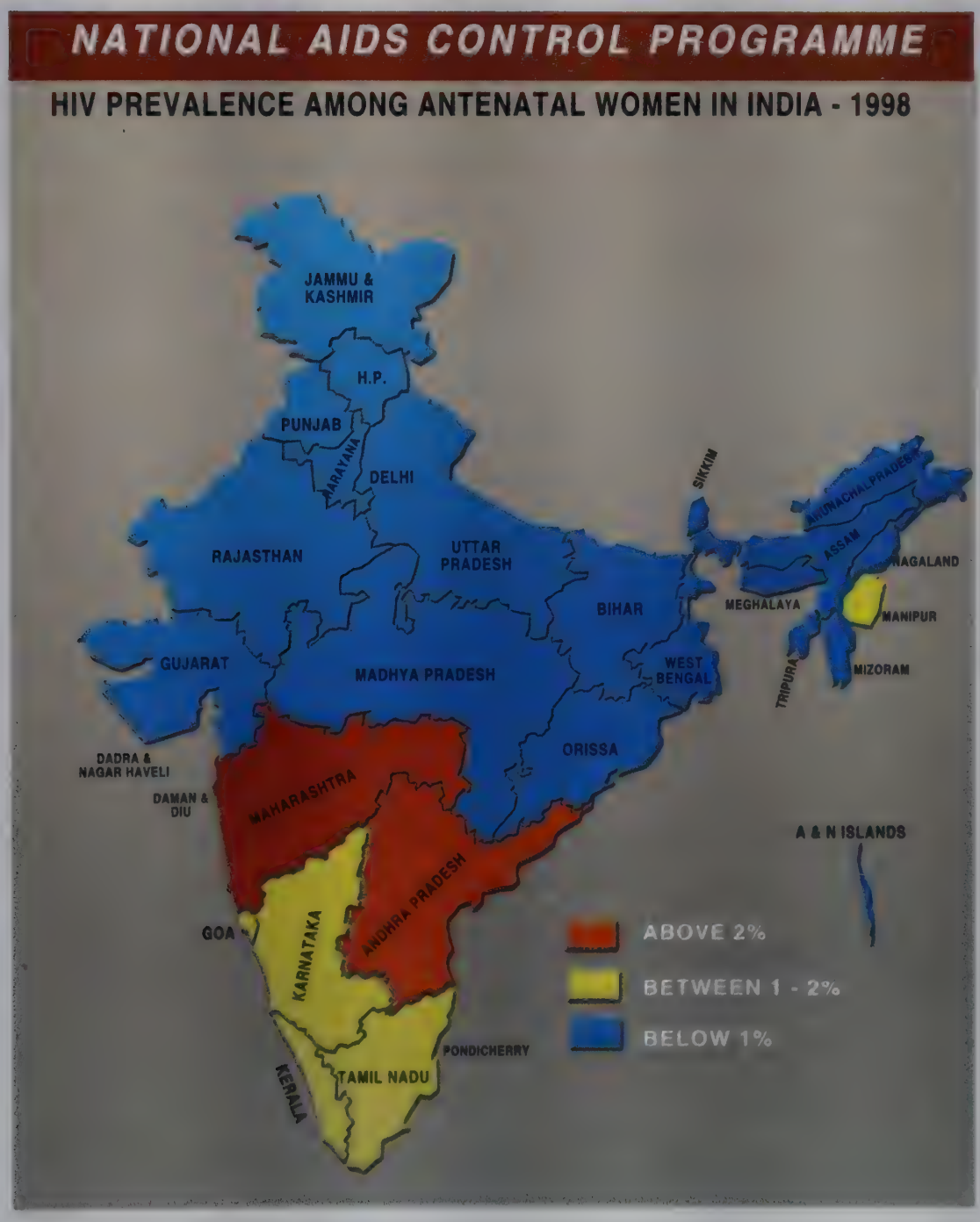


Fig. 1.6 HIV Prevalence among Antenatal Women in India - 1998

1.3 EPIDEMIOLOGICAL FEATURES

1.3.1 Agent factors

i) **Agent:** HIV, the virus which causes AIDS is a lentivirus, one of sub family of retroviruses. They have a unique enzyme, reverse transcriptase, which copies the viral ribonucleic acid (RNA) into deoxyribonucleic acid (DNA), which eventually integrates into host cell chromosome. Hence, HIV persists within cells for years and cannot be eradicated from host cells with any of the currently available anti-retroviral drugs. There are two type of HIV; HIV-1 and HIV-2. Both have many strains.

ii) **Reservoir of infection:** People harbouring HIV in their body are the reservoir of infection. They may be asymptomatic healthy carriers or full blown AIDS cases. According to latest observations in developed countries, 50% of people with HIV infection are likely to develop AIDS within 10 years after first becoming infected. In developing countries the interval between infection and disease is probably shorter.

iii) **Source of infection:** Contaminated blood, semen, vaginal fluids are the prime source of infection. Contaminated breast milk may be source of infection for transmission from mother to child. Saliva and tears are not considered to be epidemiologically important sources of infection.

1.3.2 Host Factors

i) **Age and sex:** Young people are disproportionately affected by HIV and AIDS. Around half of the new HIV infections are in people aged 15-24 years, the range in which most people start their sexual lives. In North America, Europe and Australia 70 percent cases are homosexuals or bisexual men. In Africa the picture is very different. AIDS occurs almost as frequently among females as males.

ii) **High Risk Groups:** HIV prevalence in certain populations is an important factor in determining on which target populations the programme's efforts and resources should focus. If estimated level of infection in targeted populations, such as injecting drug users is high, it is more likely that they will infect each other and also their sexual partners. So, this population will need to be focused for targeted intervention. When prevalence is high in a country, child bearing age becomes more at risk as also the population receiving blood transfusions.

a) **Presence of STD:** There is strong evidence that men and women with genital ulcer disease or urethral discharge are at increased risk of acquiring and transmitting HIV. If there is data suggesting high prevalence of STD in a population, this would be an influencing factor for increased risk of HIV infection in the community.

b) **Frequency of exposure:** The probability that a person has become infected with HIV sexually is, in general, proportional to the frequency of unprotected sex acts and number of high risk partners with whom the person has had sexual contact in recent years.

c) **Mixing pattern of population:** The way (risk) behaviours are distributed among groups of people can be uneven. For example, injecting drug users might only share injecting equipment within their own groups but have sexual partners that are both within and outside their identified groups.

d) **Immunity:** It was found that the AIDS victims had normal B cell function, that is their antibody levels were normal or elevated. But their antibodies were of non neutralizing variety which have no demonstrable effect on the virus. However, their T cell function was far from normal. In a healthy immune system, specialized T-cells called "helper T cells" (CD4) assist B cells and antibodies to fight infection while their counter parts, "suppressor T cells" inhibit this activity. Healthy individuals have twice as many helper cells as suppressor cells. In AIDS patients, the ratio is reversed. One of the most striking features of the immune system of patients with AIDS is profound lymphopenia with total lymphocyte count often below 500/c.mm.

1.4 POLITICAL AND CULTURAL FACTORS

i) **Acceptability of certain indigenous sexual practices:** Certain indigenous sexual practices may be culturally acceptable and these may contribute to HIV transmission. For example, man having sex with man (MSM) may be a common accepted practice but contributes to HIV transmission. Also, the national AIDS programme may not be able to promote certain safer sexual practices if they are not culturally acceptable.

ii) **War and civil disturbance:** These limit the regular importation of commodities, such as STD treatment drugs, condoms and HIV testing kits. This is not only logistic and supply disruptions but also due to profound changes in behaviour pattern, dislodging of families and persons etc. Radio and television messages promoting safer sex, condom promotion, etc. may not go as planned.

iii) **Limitations on interventions:** These hinder the progress of prevention interventions such as distribution of condoms to youth or commercial sex workers (CSWs).

iv) **Social unacceptance of condoms:** This may be a determinant of risk in certain populations. Such populations are at high risk of transmission through unsafe sexual practices.

v) **Women's status:** May limit women's ability to practice safer sex, for example, women might not be in a position to choose or make decisions such as condom use with their partners. If a woman is economically dependent on her partner, it is especially difficult for her to influence him to use condoms.

vi) **National policies:** That serve as barriers to the implementation of important interventions for example, restricted availability of needles and syringes would limit the usefulness of an intervention to promote safe drug injection practices.

vii) **Norms and practices:** Can increase the risk of becoming infected in certain populations. For example, sharing needles to "belong" to a group of drug injectors might be a common ritual that needs to be considered before designing prevention interventions for the drug injecting community. Also, it may not be culturally acceptable to discuss homosexuality.

viii) **Culture and ethnic practices:** Such as circumcision in males and females, tattooing, and scarification may be well accepted but can also contribute to the risk of becoming infected in certain populations because of use of poorly sterilized piercing equipment.

ix) **Marginalised population:** Economically depressed populations may never be able to benefit from prevention efforts because the social system refuses to recognise them. Example of such populations are CSWs and injecting drug users.

1.5 SOCIAL AND ECONOMIC FACTORS

i) **Low literacy:** May limit access to written, risk reduction information.

ii) **Urbanization:** For economic reasons many people may move to the larger cities, where they may indulge in high risk behaviours such as commercial sex and injecting drug use etc.

iii) **Imprisonment:** May restrict men's access to women and encourage men to have sex with men.

iv) **High mobility:** Certain target population may be highly mobile and increase the geographic spread of HIV transmission. For example, truck drivers may increase the spread by engaging in sex with CSWs at several truck stops.

v) **Migration and separation from families:** Industries such as fishing, trucking and mining may force people to travel to another country or region of the country to find work. The resulting separation from families and situations may drive them to seen commercial sex and casual sex.

vi) **Drug use:** Drug use may impair judgement and limit the ability to practise safer sex.

vii) **Alcohol use:** Alcohol use may impair judgement and limit the ability to practise safer sex.

1.6 MODE OF HIV TRANSMISSION

Epidemiological studies throughout the world have shown three modes of HIV transmission.

i) **Sexual intercourse:** Whether heterosexual or homosexual, is the major route of transmission. HIV can be transmitted through any individual act of unprotected sexual intercourse that is any penetrative sexual act in which a condom is not used where one partner is infected with HIV. The risk of becoming infected through an act of unprotected sexual intercourse depends on four main factors:

The likelihood that the sex partner is infected: The probability that a person has become infected with HIV is in general proportionate to the number (frequency) of unprotected sex acts and the number of high risk partners with whom the person has had sexual contact in recent years.

The type of sex act: All unprotected acts of sexual penetration (anal, vaginal, oral) carry a risk of HIV transmission because they bring sexual secretions directly into contact with exposed mucous membrane. Injury to the mucous membrane of the rectum, the vagina or the mouth may help the virus to enter into the blood stream. "Receptive" partners are thus at a greater risk than "Insertive" partners in acts of intercourse. However, HIV can be transmitted even through unbroken mucous membrane.

The amount of virus present in the blood or sexual secretions (semen, vaginal or cervical secretions) of the infected partner: Individuals with HIV infection become more infectious as they progress to HIV related diseases and AIDS. There is also an early period of high infectiousness around the time of seroconversion.

The presence of other sexually transmitted diseases and/or genital lesions in either partner: HIV can be transmitted sexually even when neither partner has any of the other

sexually transmitted diseases. However, there is strong evidence that men and women with genital ulcer disease or urethral discharge are at increased risk of acquiring and transmitting HIV.

ii) **Blood borne infections:** HIV infected blood, blood products, transplanted organs or tissues and the use of improperly sterilized needles and syringes that have been in contact with infected blood can transmit HIV. This is the most efficient way of transmission of HIV. Even a small transfusion of infected blood results in virtually 100% seroconversion.

iii) **HIV infected woman can transmit HIV to her foetus or infant before, during, or after birth:** A pregnant women with HIV infection has an approximately 30% chance of passing the virus to her foetus or newborn baby. There is evidence that infection can occur as early as the first 12-15 weeks of gestation. 60% of perinatal infections are in utero or during the birth process. It is estimated that 40% of perinatal infections occur through breast feeding.

1.7 NATURAL HISTORY OF HIV/AIDS

People infected with HIV are both infected and infectious for life, even when they look and feel healthy, they can transmit the virus to others. The signs and symptoms of infection with HIV are varied and complex. Four stages of HIV infection can be described

i) **Primary infection:** Infection with HIV results in rapid proliferation of the virus in blood and lymph nodes. The infected person may experience a seroconversion illness, which usually resolves within weeks. The CD4 cell count declines rapidly before virus is controlled by the immune system, whereupon the count returns to near normal.

ii) **Early immune deficiency (CD4 cell count > 500/ml):** During this phase the immune system has controlled the virus, which is largely restricted to lymphoid tissue. In this phase, damage inflicted by the virus is limited to the regenerative capacity of the immune system and people with HIV are usually without symptoms.

iii) **Intermediate immune deficiency (CD4 cell count 200-500/ml):** Viral replication is very high and CD4 cell turnover is rapid. Subtle signs and symptoms indicating compromise of immune system begin to appear.

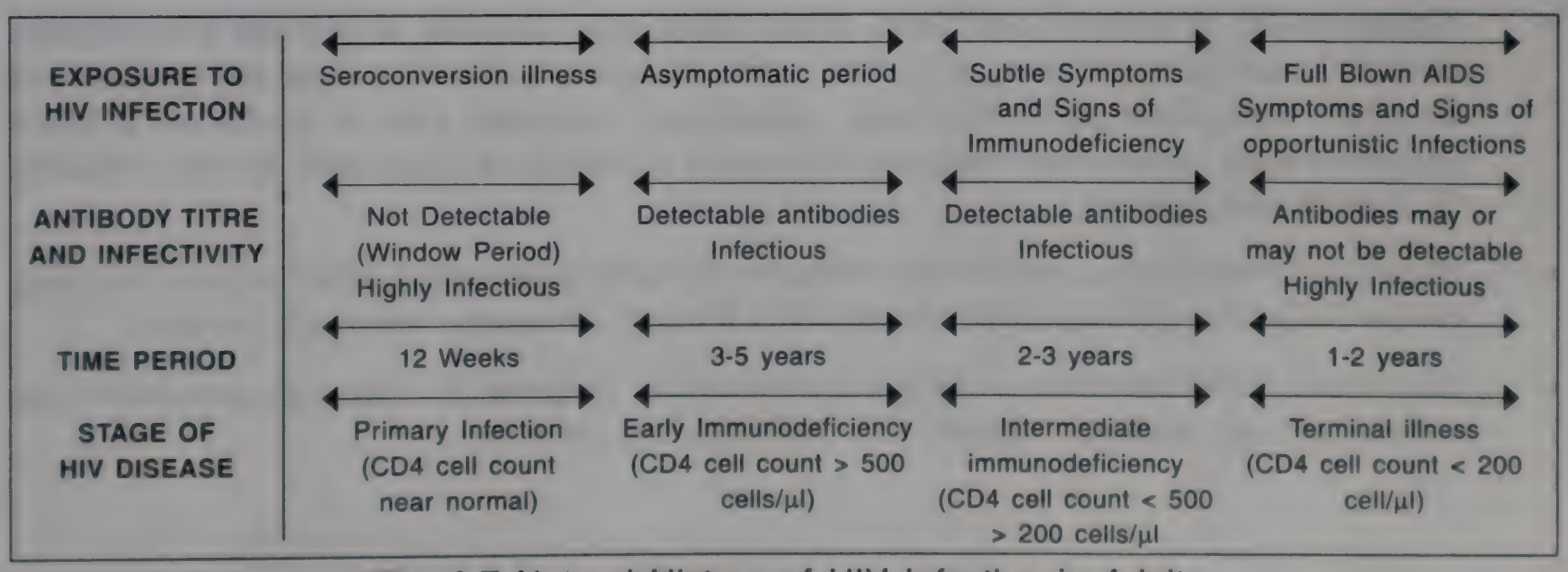

EXPOSURE TO HIV INFECTION
Seroconversion illness
Asymptomatic period
Subtle Symptoms and Signs of Immunodeficiency
Full Blown AIDS Symptoms and Signs of opportunistic Infections
ANTIBODY TITRE AND INFECTIVITY
Not Detectable (Window Period) Highly Infectious
Detectable antibodies Infectious
Detectable antibodies Infectious
Antibodies may or may not be detectable Highly Infectious
TIME PERIOD
12 Weeks
3-5 years
2-3 years
1-2 years
STAGE OF HIV DISEASE
Primary Infection (CD4 cell count near normal)
Early Immunodeficiency (CD4 cell count > 500 cells/μl)
Intermediate immunodeficiency (CD4 cell count < 500 > 200 cells/μl
Terminal illness (CD4 cell count < 200 cell/μl)

Fig. 1.7 Natural History of HIV Infection in Adults

iv) **Advance immune deficiency (CD4 cell count < 200/ml):** The virus which proliferates throughout the body overcomes the immune system. Major opportunistic infections and malignancies become increasingly common and require increasing medical intervention. **Fig. 1.7** depicts the natural history of HIV/AIDS.

NATIONAL AIDS CONTROL PROGRAM

The National AIDS Control program was launched in India in 1987. In 1992, Government of India negotiated an IDA Credit of US$ 84 million to support the implementation of a 5 year HIV/ AIDS Control project from September 1992 to September 1997. The project was later extended upto 31st March, 1999. The objectives of the project were:

1. To slow the spread of HIV
2. To decrease morbidity and mortality associated with HIV infection
3. To minimise socio-economic impact resulting from HIV infection

A National AIDS Control organisation was set up with a Project Director in the rank of Additional Secretary to Government of India and supporting technical and administrative staff. A National AIDS Control Board was set up under the chairmanship of Secretary (Health) to review policies, expedite sanctions, approve purchase of equipment and award contracts to private agencies. A National AIDS Committee was constituted under the Chairmanship of Minister of Health & Family welfare for effective intersectoral coordination in implementing the program. State AIDS Cells were established in the 25 states and 7 union territories with technical and administrative powers for implementing the scheme.

Achievements of the national AIDS control program in India

- A scientific case reporting system has been set up with 65 sero-surveillance centres spread throughout the country. A sentinel surveillance system was introduced to monitor the trend of the infection with 55 sentinel sites which were increased to 180 in 1998.
- Control of sexually transmitted diseases by strengthening and establishing 504 STD clinics and 5 regional STD research training centres.
- Establishment of National and State Blood transfusion Councils to oversee the progress made in blood safety program. 815 blood banks at various levels have been modernised and 40 blood component separation units established. Complete ban on professional blood donations w.e.f 1st January 1998 and mandatory screening of blood units for HIV, Hepatitis B, Syphilis and malaria.
- Scaling up of awareness and social mobilisation by using mass media, advocacy and involving NGOs/CBOs. Sensitising university Students through University Talk AIDS program
- Successful implementation of targeted intervention projects for high risk population like commercial sex workers, truckers and injecting drug users etc.

NATIONAL AIDS CONTROL PROGRAM PHASE II (NACP-II)

The 2nd National AIDS Control Program (AIDS II project) will shift focus from raising awareness to changing behaviour through interventions, particularly for groups at high risk of contracting HIV. The project would support decentralisation of service delivery to the states and municipalities and a new facilitating role for NACO.. The project will help to protect human rights by encouraging voluntary counselling and testing and discourage mandatory testing.. The project would support structured and evidence based annual reviews and ongoing operational research. The project would encourage management reforms to bring about 'ownership' of the program among the states, municipal corporations and other implementing agencies.

NACP II has two key objectives namely, i) to reduce the spread of HIV infection in India and ii) strengthen India's capacity to respond to HIV/AIDS on a long term basis.

Chapter 2

VIROLOGY

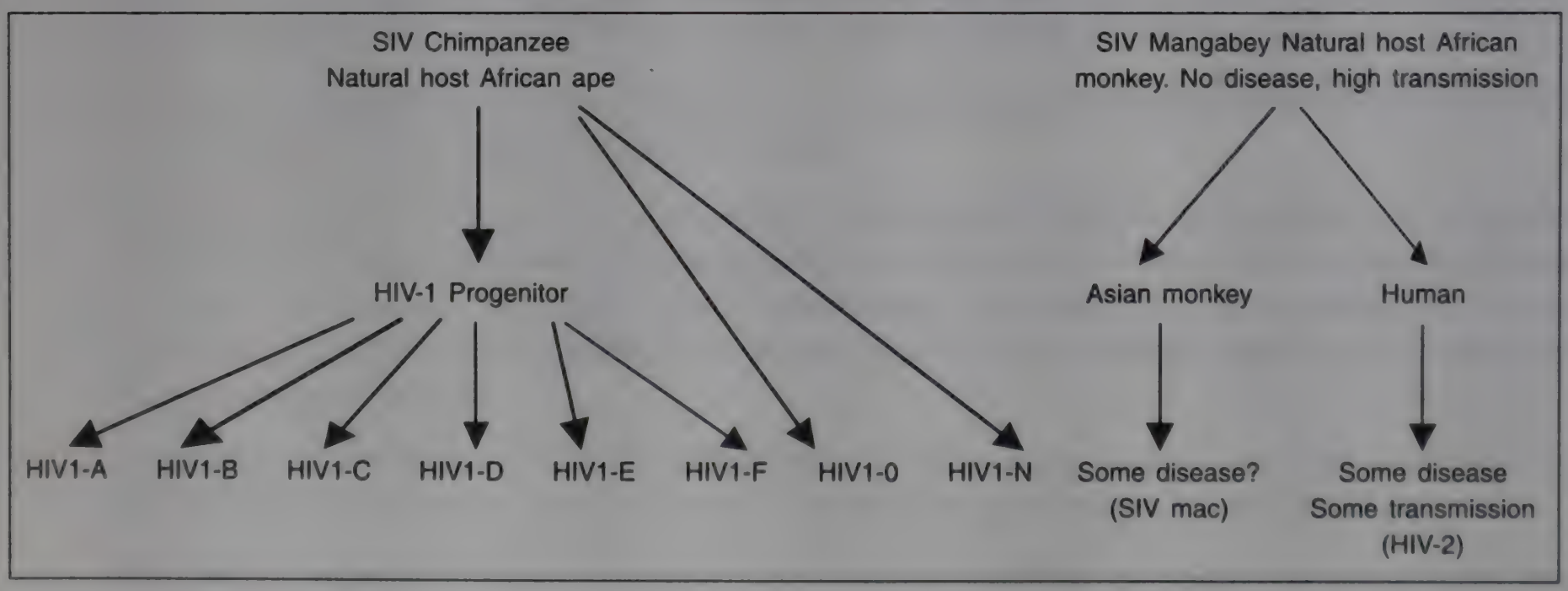
2.1 INTRODUCTION

It is very important to understand the causative agent of AIDS (Acquired Immunodeficiency Syndrome), the agent which has caused a pandemic and a disease which has enormous social, economic and behavioural impact on individuals, families, communities and the whole world. AIDS has shattered the global economy with no successful treatment and vaccine in sight.

2.2 HISTORY AND ORIGIN

AIDS was officially recognised for the first time in June, 1981 at the Centres for Disease Control, U.S.A. in previously healthy homosexual men dying with Pneumocystis carinii pneumonia and candidiasis. Since then AIDS has been reported from all the continents. The virus causing AIDS was independently identified by a team of French scientists led by Dr. Luc Montagnier of Pasteur Institute and American scientists lead by Dr. Robert C. Gallo of National Cancer Institute in 1983. The virus has been called by different names LAV i.e. Lymphadenopathy Associated Virus by the French and HTLV III i.e. Human T Lymphocytotropic Virus type III by the Americans. The International Committee on Nomenclature of Viruses named it the "Human Immunodeficiency Virus" (HIV) and to date two types, HIV-1 and HIV-2 are identified. Probable origin of HIVs is depicted in **table 2.1**.

Table 2.1 Probable Origin of HIV



2.3 CLASSIFICATION

Human immunodeficiency viruses (HIV) belong to the class Retroviruses and family Lentivirinae. Two types are recognised HIV-1 and HIV-2. Both differ in geographical distribution,

biological and molecular characteristics and extent of transmissibility. These viruses store their genetic information as ribonucleic acid (RNA). RNA must be converted to DNA by a special enzyme reverse transcriptase. HIV-1 has 3 groups, HIV-1 major group (HIV1-M), outlier (HIV1-O) and HIV1-N group. The strains of HIV-1 isolated from people in U.S.A. and Europe are genetically diverse from strains isolated in Africa and Asia. HIV-1 major group can be further classified into subtypes or clades designated A through K. Such subtypes have envelope gene sequences that vary by 20% or more between subtypes. The subtypes differ in geographical distribution, biological characteristics and major mode of transmission etc. HIV-1 subtypes O and N are more distant to all other HIV-1 subtypes but less so compared to HIV-2. So these are classified under HIV-1 only and have limited distribution in West Africa. HIV-2 has also been reported from other countries and this also comprises of heterogenous group of viruses. **Table 2.2** shows the distribution of common subtypes.

Table 2.2 HIV-1 Major Subtypes

Major epidemic pattern	Geographic Region	HIV-1 Subtype A	B	C	D	E
Heterosexual hosts	Sub-Saharan Africa	+	−	+	+	+
	South East Asia	−	−	−	−	+
	India	+	+	+	−	−
Intravenous drug users and homosexual hosts	North America	−	+	−	−	−
	Western Europe	−	+	−	−	−
	South East Asia	−	+	−	−	−
	India	−	+	+	−	−

2.4 STRUCTURE

HIV is 120 nm icosahedral, enveloped, RNA virus. HIV comprises of an outer envelope consisting of a lipid bilayer with uniformly arranged 72 spikes or knobs of gp 120 and gp 41. Glycoprotein (gp) 120 protrudes out on the surface of the virus and gp 41 is embedded in the lipid matrix. Inside is the protein core surrounding two copies of RNA. Core also contains viral enzymes reverse transcriptase, integrase and protease, all essential for viral replication and maturation. Proteins p7 and p9 are bound to the RNA and are believed to be involved in regulation of gene expression (**Fig. 2.1**).

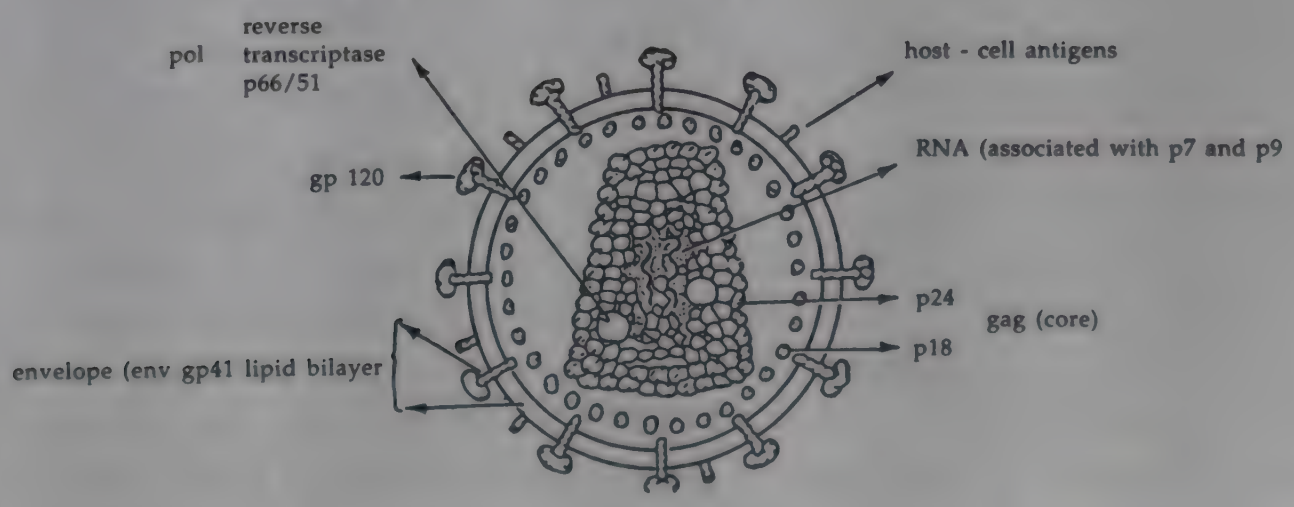


Fig. 2.1 Representation of the structure of the human immunodeficiency virus

2.5 GENETIC STRUCTURE

The genetic structure of virus contains both highly conserved and highly variable regions. The high variability of the virus accounts for drug resistance and evasion from immune response. This also poses problems for development of a successful vaccine. In an infected individual, quasispecies of a particular viral subtype may be found on account of constant variability.

HIV has structural and regulatory genes coding for structural and regulatory products, respectively. Structural genes direct the synthesis of physical components of the virus and are also responsible for viral size, shape, structural integrity and its compartmentalization in host cell. The regulatory genes direct synthesis of proteins that effect the synthesis of viral components and viral replication (**Fig. 2.2**). Structural genes are gag pol, env and others shown in the figure are the regulatory genes. HIV also has some accessory genes.

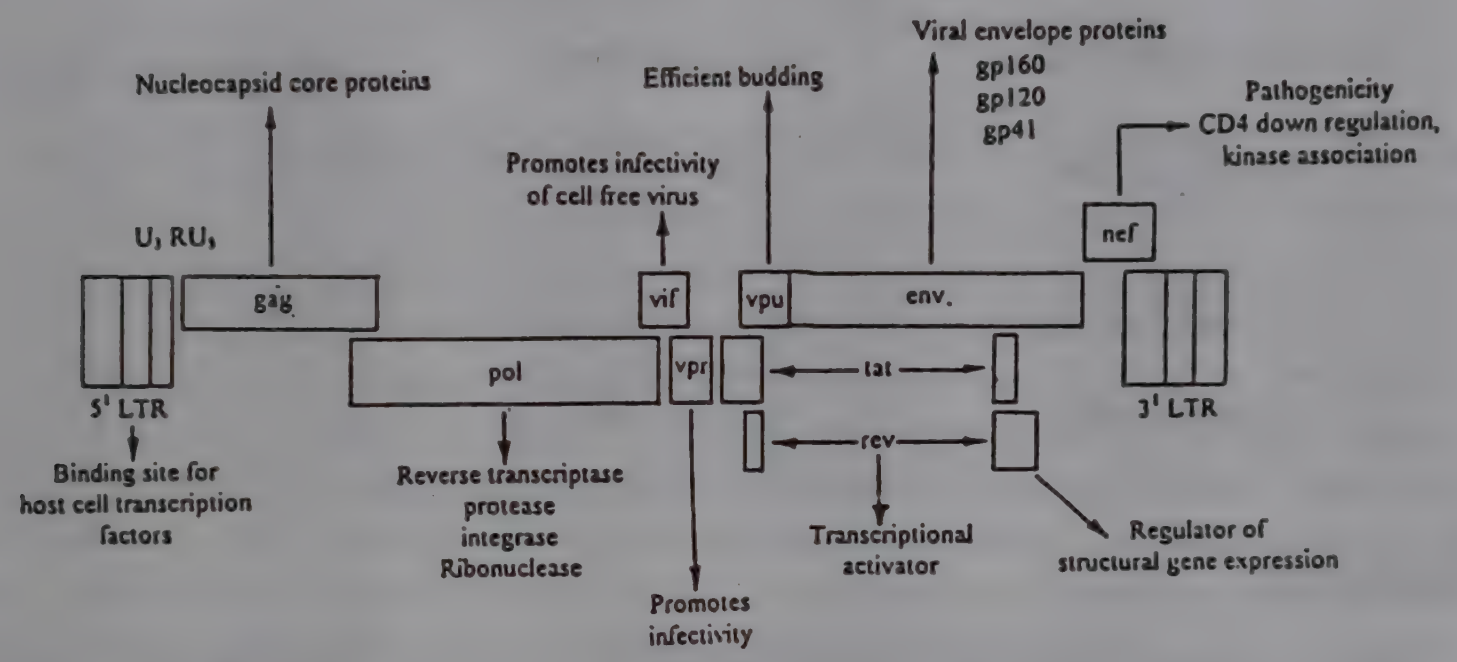


Fig. 2.2 Structure and functions of HIV-1 genome

2.6. REPLICATION

Glycoprotein 120 of HIV binds to a receptor/receptors on HIV permissive host cell. Predominant receptor is the CD4 molecule present on T lymphocytes and macrophages, though others such as galactosyl ceramide (gal C) have also been proposed. Receptors are molecules (proteins and or glycoproteins) present on the surface of host cells which facilitate the attachment and entry of viruses in to the cell. Entry of virus into the host cell requires certain cellular coreceptors/factors e.g. CCR-5, CXCR-4, CCR-2 and CCR3 etc designated collectively as cell infectivity factor (CIF). CIF may be a coreceptor or enzyme helping in virus interaction with host cell. Most convincing candidate is the chemokine receptor related protein, fusin (CXCR-4). Once the gp41 of the virus fuses with the host cell membrane the capsid is uncoated and a ribonucleoprotein complex capable of reverse transcription is formed. During the process of reverse transcription cDNA is formed under the effect of viral enzyme, the reverse transcriptase. Reverse transcription is inefficient in quiescent cells suggesting the involvement of host components in the process. The nucleoprotein complex formed after transcription comprises of linear double stranded DNA, the gag matrix (MA) protein, the accessory vpr protein and the viral integrase (IN). This is called preintegration complex and is transported into the host cell nucleus. IN mediates a complex series of

enzymatic steps and integration occurs at cellular loci with open chromatin structure. Integration probably is an essential step for viral replication. The integrated virus is called provirus. The virus may not be expressed in many cells and is considered latent. Virus expression can be stimulated by many viral, cellular and exogenous factors. Other, coexistent viral infections e.g. CMV, herpes virus etc. can make the non permissive cells permissive. Maturation of virus also takes place after virus assembly and budding.

2.6.1 HIV Replication – summary

- gp 120 binds to host cell receptors
- Reverse transcription
- Proviral DNA synthesis
- Integration with host cell DNA
- Viral proteins synthesis
- Virus assembly and budding
- Maturation of core-proteins

2.7 HETEROGENEITY/GENETIC DIVERSITY

Studies conducted on different isolates of HIV revealed that the HIV is highly heterogeneous in a variety of biological, serological and molecular features. These include:

- Level of virus production
- Replication kinetics in vivo and in vitro
- Cellular tropism (types of cells infected)
- Cytopathicity
- Syncytium forming ability
- Latency and inducibility
- Sensitivity to neutralizing/enhancing antibodies
- Genetic structure.

There are two main types HIV-1 and HIV-2. HIV-2 which was isolated in Portugal from patients with AIDS who had come from West Africa showed sequence difference of more than 55% from earlier isolated strains of HIV, so was designated a different type status. HIV-2 sequences are closer to SIV than HIV-1. HIV-1 and to a lesser extent HIV-2 are undergoing mutation during each replicative cycle. Quasispecies are being thrown constantly into circulation of the infected individual. HIV-1 strains from different geographical areas are different genetically (>20% sequence differences). Three groups major, outlier (O) and N have been identified for HIV-1 type. Major group has been classified into different subtypes/clades A through K on the basis of genetic, geographical, molecular and biological differences as above. Each subtype again comprises of genetically heterogeneous strains on account of high rate of variability of the virus even in the same host. Heterogeneity of virus has implications for development of a successful vaccine and a therapeutic agent.

2.8 HUMAN CELLS/CELL LINES AND TISSUES SUSCEPTIBLE TO HIV

HIV practically multiplies in all cells but the extent of replication varies in different cells:

- **Haematopoietic system:** T lymphocytes, B lymphocytes, Macrophages, NK cells, Megakaryocytes, Dendritic cells, Promyelocytes, Stem cells, Thymic epithelium, Follicular dendritic cells.
- **Brain:** Capillary endothelial cells, Astrocytes, Macrophages (microglia), Oligodendrocytes, Choroid plexus, Ganglia, Neuroblastoma cells, Glioma cell lines and Neurons (?).
- **Skin:** Fibroblasts and Langerhans cells (?).
- **Bowel:** Columnar and goblet cells, Enterochromaffin cells and Colon carcinoma cells.
- **Others:** Myocardium, Renal tubular cells, Synovial membrane, Hepatic sinusoid epithelium, Hepatic carcinoma cells, Kupffer cells, Pulmonary fibroblasts, Foetal adrenal cells, Adrenal carcinoma cells, Retina, Cervix epithelium (?), Prostate, Testes, Osteosarcoma cells, Rhabdomyosarcoma cells, Foetal chorionic villi, Placental trophoblast cells.

2.9 MECHANISM OF CELL DEATH

- Increase in cell permeability due to budding of virus. Virus punches holes and kills the cell.
- Increase in cell permeability due to toxic effects of virus replication.
- Syncytia formation – involving uninfected cells.
- Apoptotic cell death of activated T cells.
- Auto-immune phenomenon involving CD4 molecule.
- ADCC i.e. antibody dependent cell cytotoxicity.

2.10 SUSCEPTIBILITY OF HIV

Fortunately HIV is a very fragile virus. It is susceptible to heat, a temperature of 56°C for 30 minutes or boiling for a few seconds kills the virus. Most of the chemical germicides used in hospital/laboratories and health care settings kill HIV at much lower concentrations. Thus 0.5% to 1% sodium hypochlorite, 70% ethanol, 2% glutaraldehyde, acetone, ether, beta propiolactone (1:400 dilution) and sodium hydroxide (40 m Mol/litre) inactivate the virus.

2.10.1 Sterilization and disinfection

Summary of methods which kill all microorganisms including HIV (details in chapter 4)

Sterilization

- Autoclaving at 121°C, 15 lbs pressure for 20 minutes.
- Dry heat 170°C for 1 hr.
- Boiling for 20-30 minutes.

Chemical disinfection

- Sodium hypochlorite: 5 gm/litre. (0.5 to 1% ordinarily, 5-10% for high organic matter content e.g. discarding tissues etc.)
- Calcium hypochlorite: 1.4 gm/litre.
- Chloramine: 20 gm/litre
  (Available chlorine 0.1%)
- Ethanol: 70%
- Formalin: 3-4%
- Glutaraldehyde: 2% for 30 minutes
- Polyvidone iodine (PVI)

2.11 TRANSMISSION

Risk factors for HIV infection include multiple homosexual or heterosexual partners; contaminated blood transfusion; injections with contaminated needles and syringes and infected mother to foetus/infant, (before, during or shortly after birth). The efficiency of transmission of HIV is determined by the amount of virus in a body fluid and the extent of contact. High concentrations of free infectious virus and virus infected cells have been reported in blood, genital fluids and cerebrospinal fluid. Breast milk and saliva yield varying numbers, whereas, other body fluids have a low viral content. High levels of virus are always associated with symptoms and advanced disease.

Saliva in adults contains some nonspecific inhibitory substances like fibronectins and glycoproteins which could prevent cell to cell transfer of virus. Thus, saliva is not a likely vehicle of transmission. Urine, sweat, milk, broncho-alveolar lavage fluid, amniotic fluid, synovial fluid, faeces and tears have been reported to yield zero or a few HIV particles. Hence, these vehicles also do not appear to be important in virus transmission.

Breast milk at the time of primary infection in a feeding mother has a high content of virus and may transmit the infection to the baby. Cerebrospinal fluid (CSF), on the other hand, also has a high content of virus particularly in individuals with neurological disease, but, CSF is not a natural source of virus transmission.

The most efficient vehicle of HIV transmission is blood (**Table 2.3**). However, the risk

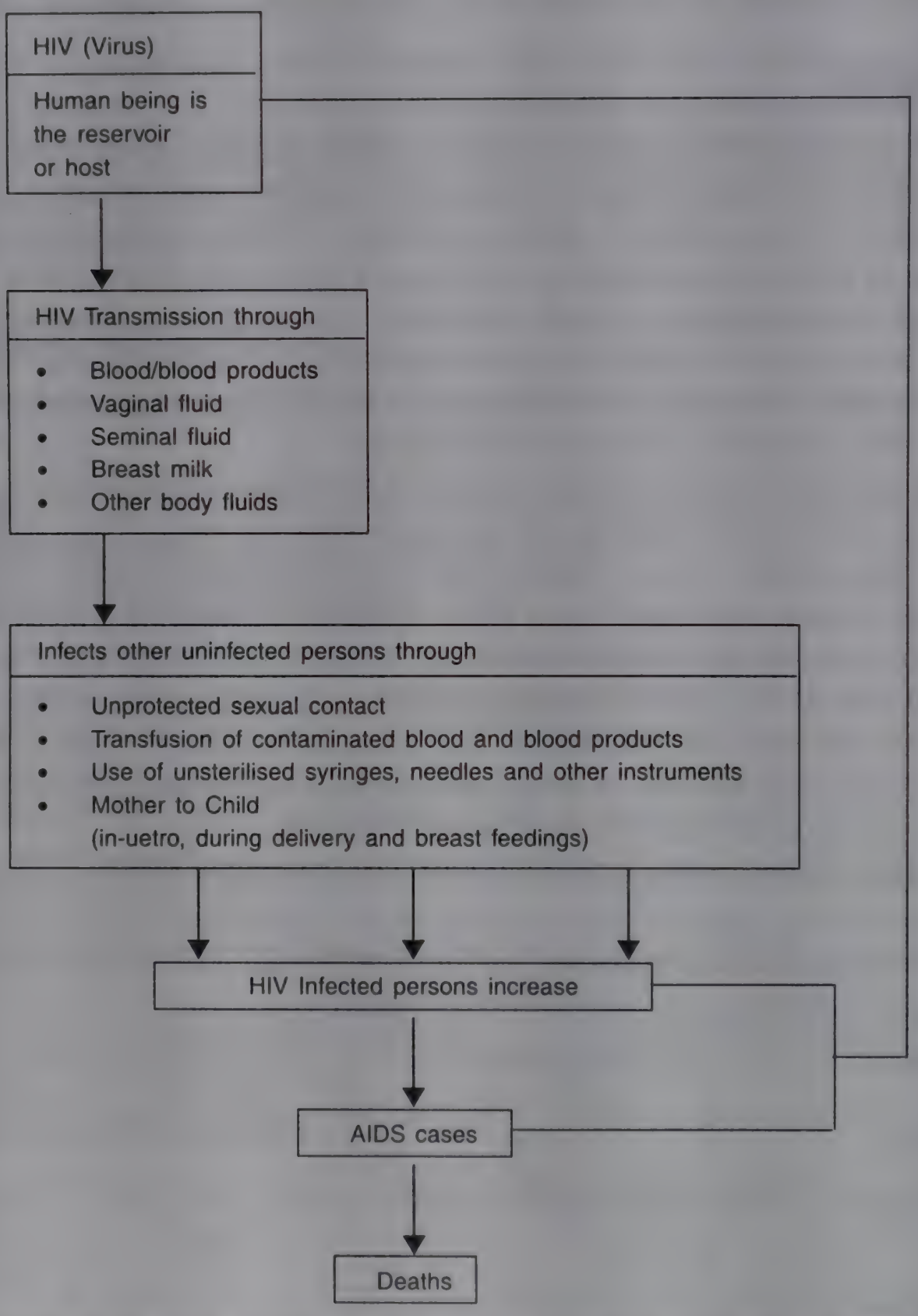
Table 2.3 Efficiency of different routes of HIV transmission and their contribution to total number of cases

Exposure Route	Per cent Efficiency (World over)	Percentage of total	
		(World over)	(India)
Blood transfusion	90-95	5	7.05
Perinatal	20-40	10	
Sexual intercourse	0.1 to 1	75	74.15 (heterosexual)
Vaginal		(60)	0.58 (homosexual)
Anal		(15)	
Injecting drugs use	0.5-1.0	10	7.3
Needle stick exposure	< 0.5	0.1	0
Others			10.92

(Percentage of total in India from NACO document entitled "Country Scenario," 1998)

of infection via blood transfusion is now extremely low due to strict HIV screening of donated blood. The most common route of transmission is unprotected, penetrative sexual contact. Different forms of sexual practices carry a variable risk gradient of acquiring HIV. Cell associated rather than free virus is responsible for disease transmission. Anal intercourse carries a high risk of transmission because of bowel mucosa which acts as a portal of entry for virus, and also because of a greater chance of injury to the mucosa. Risk to insertive partner is through infection of lymphocytes and macrophages in the foreskin or along the urethral canal. In females HIV transmission occurs when infected cells in the semen gain entry into the female genital tract, and infect the resident lymphocytes, macrophages and probably the uterine epithelial cells.

The transmission from infected mother to child appears to occur in 20-40% children born to HIV positive mothers. The source of virus in the newborn is controversial. HIV


HIV (Virus)
Human being is the reservoir or host
HIV Transmission through
• Blood/blood products
• Vaginal fluid
• Seminal fluid
• Breast milk
• Other body fluids
Infects other uninfected persons through
• Unprotected sexual contact
• Transfusion of contaminated blood and blood products
• Use of unsterilised syringes, needles and other instruments
• Mother to Child
(in-uetro, during delivery and breast feedings)
HIV Infected persons increase
AIDS cases
Deaths

Transmission of HIV through casual contact, sharing utensils, lavatories and through insect bites has not been documented so far.

Fig. 2.3 Transmission cycle of HIV

infection can occur via amniotic fluid, genital secretions, maternal blood and through the breast milk. Transmission to the baby can occur in-utero, and during or after delivery.

Transmission of HIV infection to Health Care Workers (HCW) is extremely uncommon. Pooled data from 20 prospective studies suggests that risk associated with needle injury from HIV infected blood is approximately 0.5 per cent. Further, the risk associated with mucocutaneous contact is too low to be reliably estimated. The risk from mucosal or non-intact skin is also minimal.

Fig. 2.3 depicts the different routes of transmission of HIV.

So far, there has been no report of HIV transmission through a casual social contact, by the enteric or respiratory route, and through an insect e.g. mosquito bite. Prospective studies offer a conclusive evidence that family members and close household contacts of HIV infected individuals are not at risk of acquiring HIV infection through casual human contact (shaking hands, kissing and by sharing of utensils, toilet, linen, bed etc.) or by providing routine nursing care.

Chapter 3

IMMUNOPATHOLOGY

3.1 INTRODUCTION

To understand the effects of HIV infection a reminder of body defence is a must. T Lymphocytes and B Lymphocytes together defend the body against all kinds of assaults the body is exposed to, at all times. Both T and B cells migrate from bone marrow, but T cells mature in thymus, where they develop special functions. Both kinds of lymphocytes when activated by antigen, multiply and change. B cells develop into plasma cells, produce specific antibodies which trap and kill microorganisms (bacteria mostly). This is "humoral immunity". T lymphocytes are responsible for cell-mediated immunity, very important defence against fungi, protozoa, mycobacteria and viruses. T Lymphocytes include helper T cells (CD4) and cytotoxic/killer T cells (CD8). They release soluble factors lymphokines (cytokines), can regulate humoral suppressor cells and can become memory cells. So CD4 cell population is central in defending the body. It is the key cell and this is the cell HIV infects and destroys progressively. Infection with HIV irrespective of type (HIV-1 or HIV-2) subtype and route of infection leads to protracted disease and depletion of CD4 cells in most cases resulting in AIDS. The rate of progression of disease depends upon viral characteristics on the one hand and host factors on the other hand and may take from 1 year to more than 15 years.

3.2 VIRAL ENTRY

Cell free or cell associated HIV enters the body during high risk practices through any route via blood, semen and vaginal secretions from an infected person. HIV infection is facilitated by presence of ulcerative and to a lesser extent nonulcerative sexually transmitted infections. HIV immediately targets on to cells displaying complementary receptors (CD4, CCR-5 and CXCR-4/fusin) which may be CD4 cells, resident macrophages or Langerhans cells depending upon the site of exposure. Virus gp120 fits on the receptor like a lock and key system. Viral replication starts immediately after entry into the cell and dissemination occurs through circulatory and lymphoid systems.

3.3 PRIMARY HIV INFECTION

During this stage HIV and HIV infected cells reach the lymph nodes and the other lymphoid tissues, where active immune response to viral antigens occurs and at the same time intense replication of virus occurs in activated T lymphocytes. This is a paradox because lymphocytes are activated on account of infection and HIV replicates better in these activated cells. The peak in number of virus expressing cells and spread of virus throughout the lymphoid tissue precedes the increase in plasma viraemia i.e. the virus in the blood. The virus spills over from lymph nodes. These phenomena occur during the first 2-3 weeks after infection and there is intense virus spreading during this period so this is called the **stage of virus dissemination**. Clinically it coincides with "flu like illness" also known as acute HIV

disease. There is significant fall in CD4 cells and viral levels may be as high as 10^6-10^7 viral copies/ml. The next **stage is that of down regulation of viraemia**. This coincides with robust, intense immune response by the host. Both effective cell mediated immune response carried out by HIV specific cytotoxic T lymphocytes (CTL) and humoral response carried out by complement fixing and neutralizing HIV specific antibodies comes into play. The period from the entry of HIV in the host and the appearance of detectable levels of HIV specific antibodies is called **"window period"**. During this period individual is infected, is infectious to others but is seronegative i.e. HIV tests for detecting antibodies are negative. Window period ranges from 3 weeks to 3 months on average, can be longer sometimes. Both HIV specific antibodies and CTL kill the virus infected cells. As a result the viraemia drops and CD4 cells bounce back to levels slightly lower than the previous normal level. Most of the virus trapping and killing occurs in Follicular Dendritic Cells (FDC) of the lymph nodes and lymphoid tissue. This may be one reason of generalised lymphadenopathy seen in HIV disease. Appearance of neutralizing HIV specific antibodies heralds the transition from acute to **chronic stage of HIV disease**. Although the immune response succeeds in downregulating the viraemia, HIV is never completely eliminated and progression to chronic phase of HIV disease occurs in most cases. What determines the progression of the HIV disease is the quality of T cell response. This may be genetically determined.

Fig. 3.1 depicts the course of events following viral entry in the body.

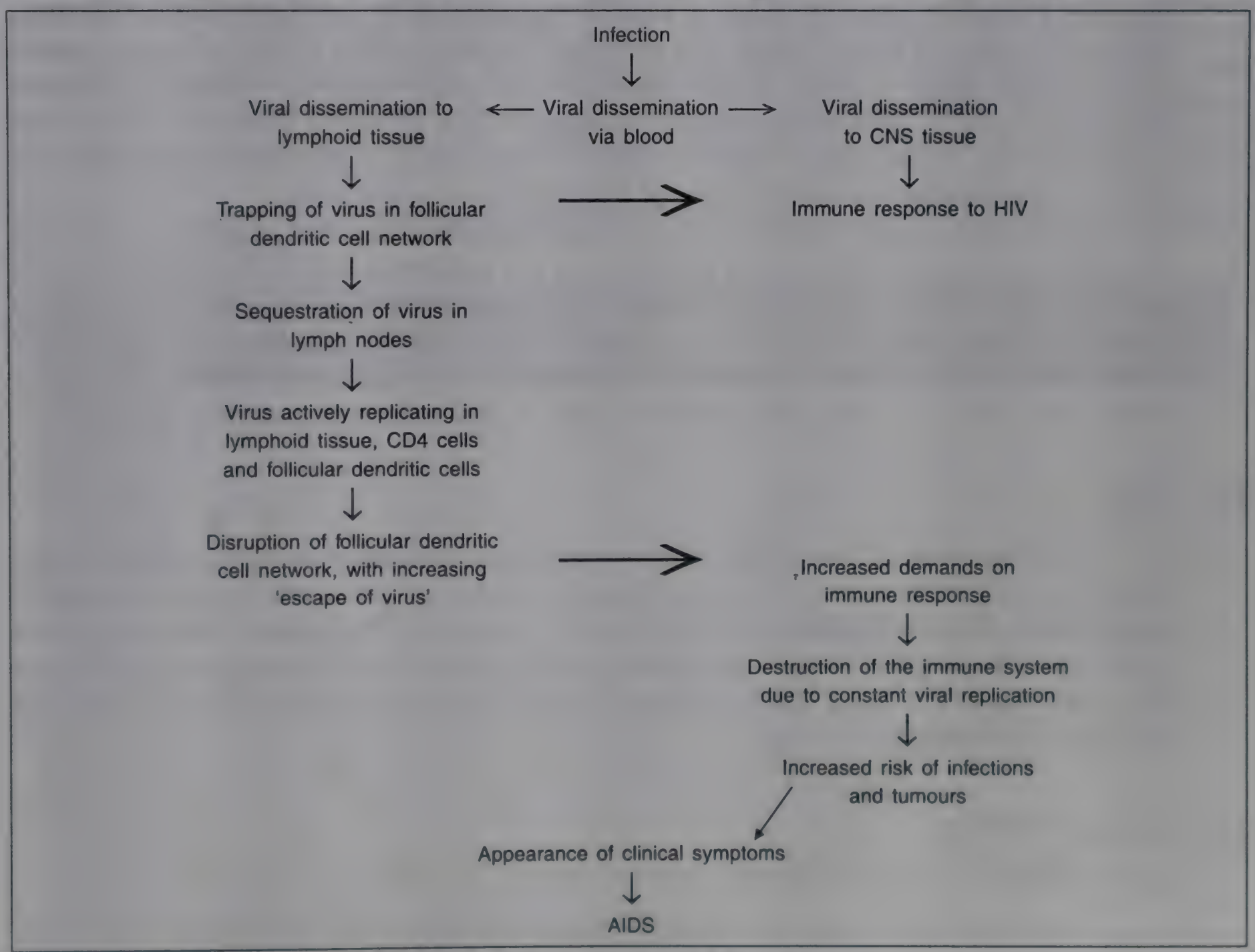

Infection
Viral dissemination to lymphoid tissue
Viral dissemination via blood
Viral dissemination to CNS tissue
Trapping of virus in follicular dendritic cell network
Immune response to HIV
Sequestration of virus in lymph nodes
Virus actively replicating in lymphoid tissue, CD4 cells and follicular dendritic cells
Disruption of follicular dendritic cell network, with increasing 'escape of virus'
Increased demands on immune response
Destruction of the immune system due to constant viral replication
Increased risk of infections and tumours
Appearance of clinical symptoms
AIDS

Fig. 3.1 Viral Pathogenesis and immune response

3.3.1 Primary infection summary

- Dissemination of HIV to lymph nodes and other lymphoid organs.
- Viraemia
- Window period
- Acute HIV disease → Acute glandular fever like syndrome / Acute meningoencephalopathy (less common)
- CTL response
- Antibody response

3.4 CLINICALLY LATENT PERIOD/CHRONIC ILLNESS

This stage is marked by disappearance of symptoms of acute viral disease, down regulation of viraemia, CD4 cell count becomes almost normal and the neutralizing and complement (C^1) fixing virus specific antibodies appear in the blood. All virological parameters in the peripheral blood (viral RNA copies/viral load, virus expressing mononuclear cells, etc.) are very low. However, active and continuous virus replication goes on in the lymph nodes and lymphoid organs which express virus 1-3 logs higher than the peripheral blood. As long as the CD4 counts are higher than 500 cells/μl, the immune response mounted by the lymphoid tissues is effective, there is follicular hyperplasia of germinal centres indicating immune activation of lymph nodes. The important paradox to note is that cellular activation seen in lymph nodes is critical for viral replication i.e. virus replication is better in activated CD4 cells. There is gradual reduction in number of CD4 cells and increase in virus load during the long asymptomatic stage. Increase in virus load in peripheral blood indicates failure of and progressive deterioration of effective immune response.

Humoral immunity is intact during the asymptomatic stage that is specific antibodies are produced against different viral proteins, but the antibodies are not protective, are not able to interfere with cell to cell transmission and infectivity of virus on account of constant variation of virus. This period on average lasts for 8-10 years. Progressive impairment of HIV specific and nonspecific cell mediated and humoral immune responses heralds the onset of AIDS. The CD4 cell counts range between >200 to 500 cells/μl in peripheral blood.

3.5 AIDS

Advanced stage of HIV infection is characterised by increase in all virological parameters (virus load, p24 antigen etc.) in both peripheral blood and lymph nodes. Lymphoid tissue is totally destroyed and replaced by fibrous tissue. Virus trapping by whatever lymphoid tissue remains is minimal or nil. There is profound immune suppression and opportunistic infections may prove fatal at this stage. The CD4 count is usually less than 200 cells/μl and progressively falls (**Fig. 3.1 and 3.2**).

3.5.1 Points to Ponder

- Infection with HIV is lifelong.
- Severe immunodeficiency develops in infected persons within 10-12 years on average.
- Consequent to sufficient immune damage, susceptibility to opportunistic infections and

cancers increases.

- Cancers and opportunistic infections act as surrogate clinical indicators of AIDS.
- Survival after diagnosis of AIDS is short, 1-2 years on average.

3.6 MECHANISM OF CD4 CELL DEPLETION AND DYSFUNCTION

CD4 cells are the main targets of HIV and progressive destruction of these cells is characteristic of all stages of HIV disease. CD4 cells serve as surrogate markers to monitor the progression of HIV infection. These cells can be destroyed by two mechanisms:

- Direct damage by the virus.
- Immune mechanisms triggered during the course of HIV infection.

HIV can kill cells singly or after giant cell and syncytia formation. Single cell killing occurs due to accumulation of unintegrated viral DNA and inhibition of cellular protein synthesis. Syncytium formation is induced by virulent strains of HIV in a multistep mechanism. CD4 cells expressing viral antigens on the surface attract CD4 uninfected cells and the membranes of these fuse producing giant cells and syncytia. One such HIV infected cell can eliminate hundreds of uninfected cells by syncytium formation. Glycoprotein 120 and other intracellular adhesion molecules bring about the cellular adhesion and subsequent damage.

The non virologic mechanisms which can damage/destroy CD4 cells include auto-immune mechanisms, anergy, superantigens, apoptosis (programmed cell death) and virus specific immune responses. A number of hypothesis and complex immune mechanisms have been postulated for CD4 cell depletion involving one or more of above mentioned pathways.

Fig. 3.2 depicts the kinetics of CD4 T lymphocytes during the course of HIV infection vis-a-vis viral load.

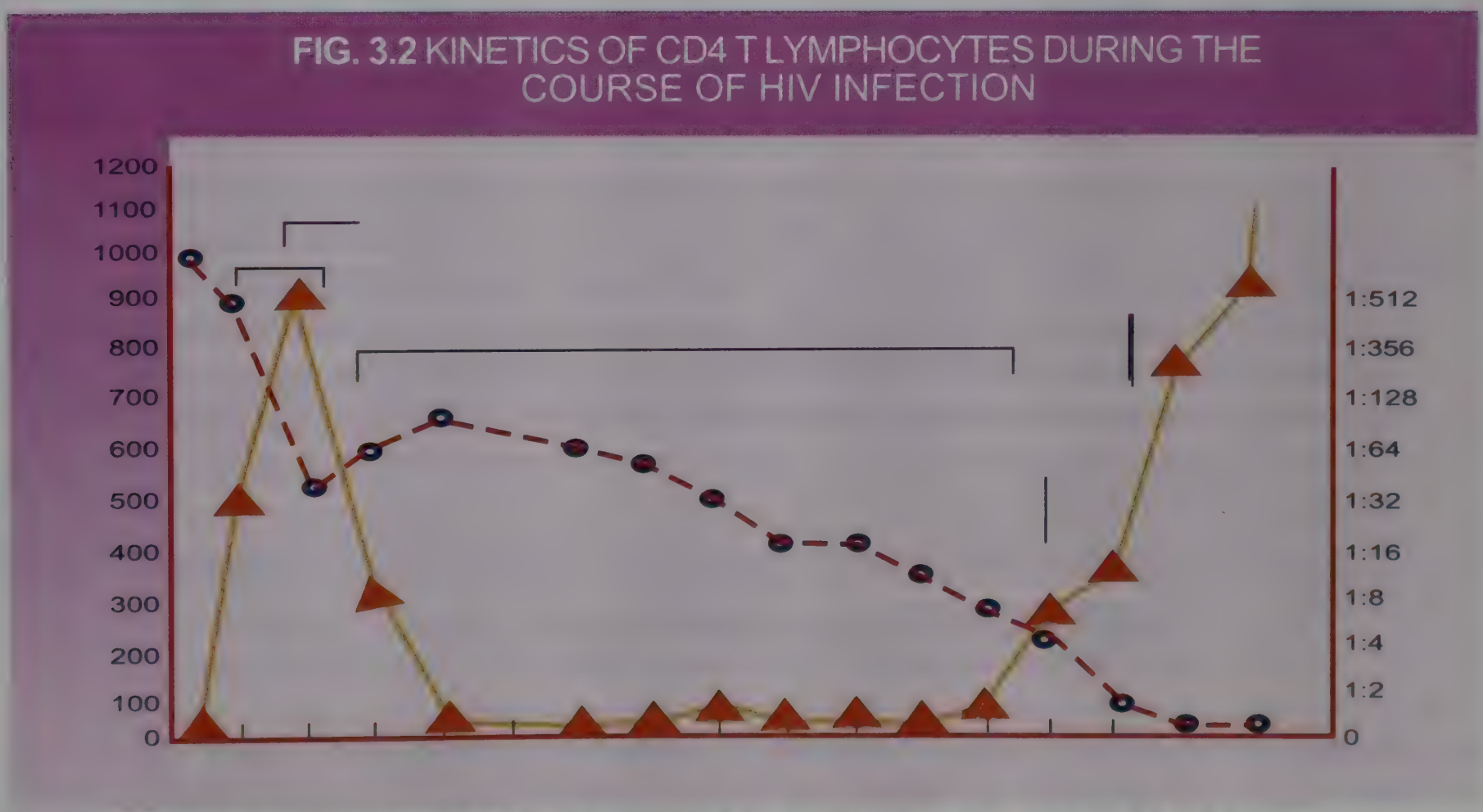


Fig. 3.2 Kinetics of CD4 T Lymphocytes during the Course of HIV Infection

3.7 THE COURSE OF PROGRESSION HIV INFECTION

Three dominant patterns of HIV disease progression have been described. These are based on the kinetics of immunologic and virologic events described above.

- 80%-90% of HIV infected are "typical progressors" with a median survival time of 10 years, approximately.
- 5% to 10% of HIV infected individuals are "rapid progressors" with a median survival time of 3-4 years approximately.
- About 5% of HIV infected individuals do not experience disease progression for an extended period of time and are called "long term non progressors" (LTNPs).

3.7.1 Typical progressors

The typical course of HIV infection includes three phases: primary infection (seroconversion), clinical latency and clinically apparent disease.

Primary phase may be totally inapparent or may be associated with acute flu like or mononucleosis like syndrome in 50% to 70% individuals. Occurs within 3-6 weeks of infection and may last for 9-12 weeks. There is high level of virus in the blood. The course of HIV disease is as described above. Progression of HIV infection to AIDS on average occurs in 8-10 years approximately.

3.7.2 Rapid Progressors

In about 5% to 10% HIV infected rapid progression to AIDS occurs within 2-3 years after seroconversion. Immune response is defective in these individuals. Levels of neutralizing and C^1 fixing HIV specific antibodies are low and CD8 cell mediated suppression of HIV replication is 'impaired'. As a result progression to AIDS is rapid in these individuals.

3.7.3 Long term non-progressors (LTNPs)

A small percentage (5%) of HIV infected individuals do not experience clinical progression of HIV and have stable CD4 cell counts over long period without any therapy. CD4 counts stay at around >500 cells/ml., cell mediated and humoral immune responses are comparatively strong in these individuals. Absolute number of CD8 cells is also persistently high in these persons. In addition the cytotoxic T lymphocytes retain their cytotoxic activity against HIV. The titer of neutralizing antibodies against HIV is also higher in these individuals. The virus specific parameters like virus load, virus replication in peripheral blood and lymph nodes are four fold to 20-fold lower in long term nonprogressors. Also the virus infecting these persons may be of low pathogenicity. Some host genetic factors may also be responsible for these LTNPs.

Fig. 3.3 depicts the progression of HIV infection in different hosts.

3.8 TYPE 1 AND TYPE 2 RESPONSES IN HIV INFECTION AND EXPOSURE

The loss of helper cell function in asymptomatic individuals is seen even before the fall in number of CD4 cells. This is evident from in-vivo and in-vitro studies. Progression of

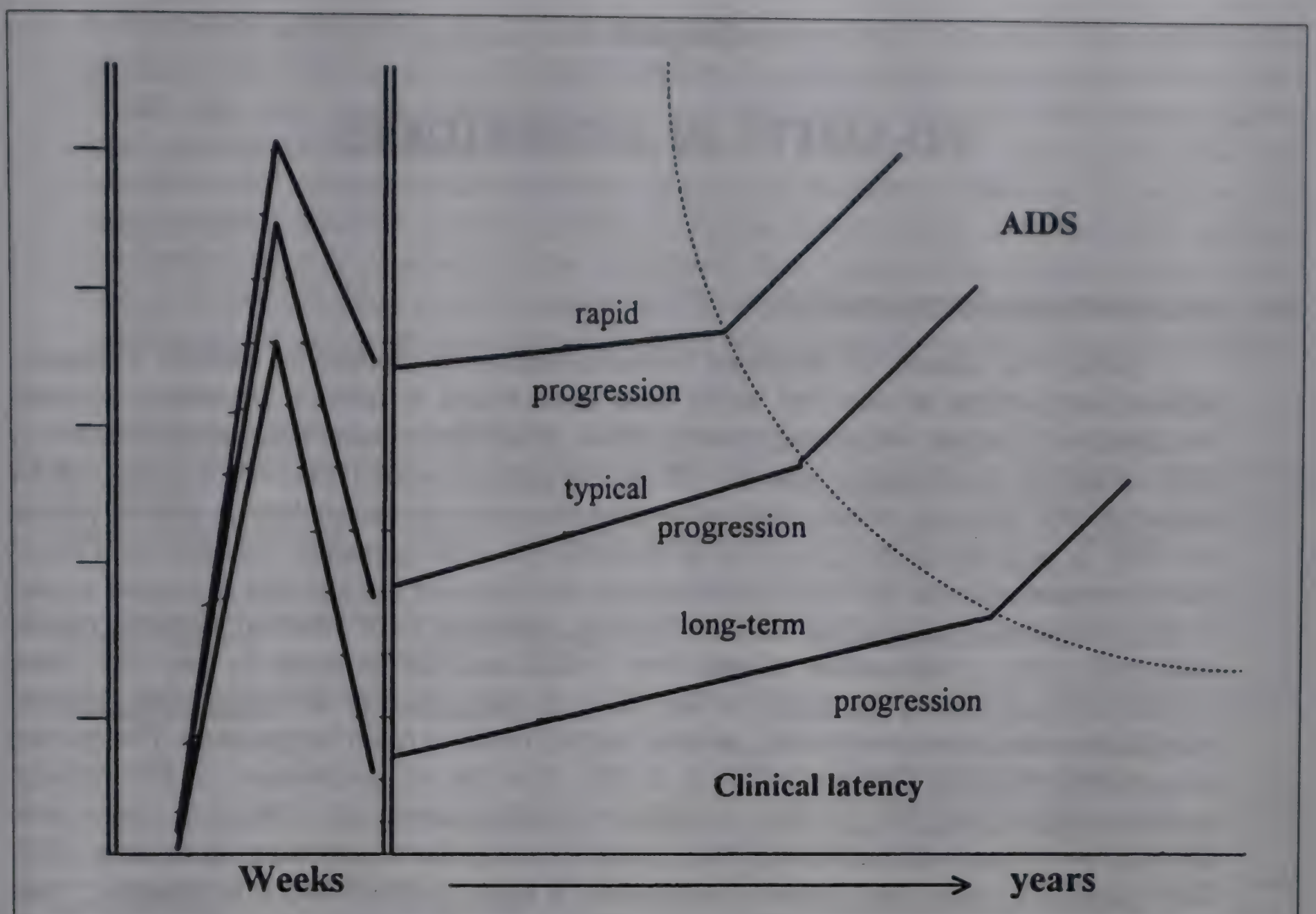


Fig. 3.3 Viral load and course of infection

HIV infection from asymptomatic to symptomatic stage and then AIDS involves shift of Th1 type responses (cell mediated immune responses) to Th2 responses (humoral immune responses).

	Th1 or Type 1	Th2 or Type 2
	Dominant cellular response	Dominant antibody response
	IFN-Y	IL-4
Cytokines which modulate these response	IL-2	IL-5
	IL-12	IL-10
	IL-15	IL-13

Cytokine induced immune dysregulation is a central component in progression to AIDS. This shift in type of immune response and consequent progression to AIDS may be on account of the fact that HIV infects Type-1 cells easily compared to Type-2 cells. The shift in immune response is associated with shift in cytokine profile also (from Type-1 to Type-2) and progression to AIDS. This change in cytokine profile results in inability of host macrophages to get rid of opportunistic pathogens resulting in opportunistic infections and neoplasms which ultimately prove fatal.

Chapter 4

BIO-SAFETY IN LABORATORIES

4.1. BACKGROUND INFORMATION

Since the advent of Acquired Immuno-deficiency Syndrome (AIDS) epidemic, extraordinary efforts all over the world have been made to prevent laboratory acquired infections with Human Immuno-deficiency Virus (HIV). The recent statistics published by CDC relative to transmission risks for HIV and Hepatitis B virus (HBV) are that the risk for acquiring HIV following needle stick prick (percutaneous exposure) from a source patient with HIV is very low (0.25-0.3%). This is because the virus generally circulates at a much lower concentration (10-100 infectious doses/ml) and because it is not able to survive as well in the environment outside the body. In contrast, acquiring HBV infection following needle stick prick from a HBV carrier ranges from 9-30% as HBV is found in very high titres (>10,000,000 infectious doses/ml) in the blood of many acutely and chronically infected individuals and can survive for long periods in dried blood at room temperature. The chance of acquiring Hepatitis C virus (HCV) is 3-10%. The risk of transmission of HIV through mucocutaneous exposure is 0.05%. In different studies carried out in the U.S., there were 52 documented sero-conversions in the context of occupational exposure up to June 1997. Out of these, 45 were percutaneous exposures, 5 were mucocutaneous exposures, 1 had both and 1 had unknown type of exposure. There were 46 exposures to blood, 1 to body fluid, 1 with unspecified fluid and three involved viral cultures in the laboratory. In all, 19 laboratory technicians had got occupationally acquired HIV. The risk of occupational HIV transmission depends on:

- Prevalence of infected individuals in the population
- The frequency of exposure to contaminated medical instrument
- The relative infectivity and concentration of the virus
- If there is an exposure to a large quantity of blood
- Deep needle stick injury
- Injury with hollow bore needles and
- Source patient who died within 60 days as a result of AIDS

In India, there is no published data available on HIV transmission due to needle stick injury/mucocutaneous exposure in health care setting. The lack of proper infrastructure, low levels of awareness about safety precautions and a certain degree of complacency amongst laboratory staff are some of the concerns for the laboratory managers.

4.2 IMPORTANCE OF BIO-SAFETY PRACTICES

There is no vaccine for prevention of HIV/AIDS, treatment is expensive and is not easily available. Hence prevention of infection is the cornerstone of control of this epidemic.

In the health care settings also, prevention is considered most important. The irrational fear of accidental infection from HIV/AIDS patients invariably influences the behaviour of the health care workers (specially those who routinely handle blood and body fluids) towards their patients and their specimens. Therefore, on the one hand, it is extremely important that the laboratory workers should be aware of the risks involved in their day to day work and apply effective infection control practices to reduce the possibility of transmission of these fatal infections to a minimum. On the other hand, they should not be prejudiced and should not show discrimination towards patients of HIV/AIDS. **Table 4.1** shows the different high risk procedures for the health care workers (H.C.W.) and the various modes of acquiring infection.

Table 4.1 Modes of exposure to blood borne pathogens in the laboratory

Lab. Procedure	H.C.W. at Risk	Source/Modes of Transmission
Collection of blood/Body fluids	Laboratory technician/Nursing staff	Needle stick injury, Broken specimen container, Blood contamination of hand with skin lesion/breach.
Transfer of specimen	Laboratory technician and transport worker	Contaminated exterior of the container requisition slip.
Processing of specimen	Laboratory personnel	Puncture of skin or contamination of skin/mucous membrane from • Contaminated work surface • Spill/splash of specimen • Broken specimen container • Faulty techniques
Cleaning/washing	Support staff	Puncture/contamination of skin from • Contaminated glassware • Sharps • Contaminated work surface
Disposal of waste	Laboratory personnel support staff	Contact with infectious waste specially sharps
Transport of specimen to distant laboratory	Transport/Postal staff	Broken/leaking container

4.3 BIO-SAFETY IN A HIV TESTING LABORATORY

Bio-safety in a HIV testing laboratory may be discussed under the following headings:

- Precautions in relation to blood and body fluids – Universal Work Precautions
- Effective use of sterilisation and disinfection
- Safe disposal of hospital waste

4.3.1 Universal blood and body fluid precautions for laboratory workers (Recommended by CDC Guidelines, 1987)

Although the risk of transmission of infection due to blood borne pathogens in the laboratory cannot be eliminated altogether because chances of accidents are there, application of universal precautions minimise these to a large extent. Universal precautions refer to precautions consistently used for all patients regardless of their blood borne infection status. Under universal precautions, blood and body fluids of all patients are considered potentially

infectious for HIV, HBV, HCV and other blood borne pathogens. Similarly, all instruments and other equipments that have come into contact with blood are assumed to be potentially contaminated with blood borne pathogens and must be properly handled, cleaned and sterilized/disinfected or safely disposed of. Universal precautions are intended to prevent accidental parenteral, mucous membrane and nonintact skin exposures of health care workers to the above blood borne pathogens in patients. Routine and/or mandatory testing of patients for HIV antibody or HBsAg is not an effective strategy for controlling the transmission of these infections in health care settings.

4.3.1.1 Body fluids to which universal precautions apply

- Blood – the single most important source of HIV, HBV and other blood borne pathogens in the occupational setting
- All other body fluids containing visible blood
- Semen & vaginal secretions –not implicated in occupational transmission
- Cerebrospinal fluid
- Synovial fluid
- Pleural fluid
- Peritoneal fluid
- Pericardial fluid
- Amniotic fluid

4.3.1.2 Body fluids to which universal precautions do not apply

The risk of HIV transmission is extremely low or negligible unless these contain visible blood. These include:

- Faeces
- Nasal secretions
- Sputum
- Sweat
- Tears
- Urine
- Vomitus

4.3.1.3 Universal precautions include:

- Barrier protection
- Hand washing
- Safe techniques
- Safe handling of sharp items
- Safe handling of specimen (blood etc.)
- Safe handling of spill of blood/body fluid

- Use of disposable/sterile items
- Safe techniques including mechanical pipetting device
- Immunisation with Hepatitis B vaccine

i) Barrier protection

Protective barriers reduce the risk of exposure of the laboratory worker's skin or mucous membrane to potentially infective materials including blood and the body fluids.

Gloves

Can reduce the incidence of contamination of hands but cannot prevent penetrating injuries by needles and other sharp instruments. Gloves **should be:**

- Worn while collecting/handling blood specimens, blood soiled items or whenever there is a possibility of exposure to blood or body fluids containing blood.
- Worn while disposing laboratory waste
- Well fitting disposable viny 1 and must be changed if visibly contaminated with blood/ breached
- Heavy duty general purpose rubber gloves for washing infected glassware/sharps. These utility gloves may be decontaminated and reused but should be discarded if they are peeling, cracked or discoloured or if they have puncture, tears etc.
- Removed before handling door knobs, telephones, pens, performing office work and leaving the laboratory.

Laboratory gowns

- Laboratory gowns or uniforms (preferably wraparound gowns) should be worn when in the laboratory and should be removed before leaving.
- Plastic aprons should be used while cleaning infected re-usables and during disposing wastes.

Facial protection

- Simple and cheap deflector masks and protective glasses may be worn if splashing or spraying of blood/body fluids is expected.

Occlusive bandage

- All skin defects e.g. cuts, scratches or other breaks must be covered with water-proof dressing before patient care.

ii) Hand washing

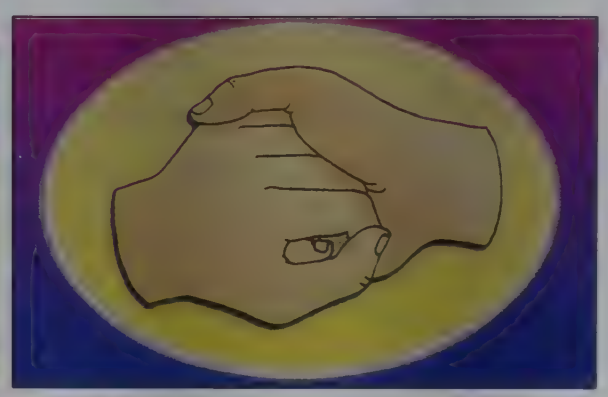
This is an ideal safety precaution and is one of the most important in preventing HIV

Fig. 4.1 Areas commonly missed in hand washing

Fig. 4.2 Steps of effective handwashing

Step 1
Wash palms and fingers

Step 2
Wash back of hands



Step 3
Wash fingers and knuckles

Step 4
Wash thumbs

Step 5
Wash finger tips

Step 6
Wash wrists

transmission in health care settings.

- Hands should be washed thoroughly in running water with soap without missing any area (**Fig. 4.1** and **4.2**).
- Washing of hands is mandatory
- immediately after contamination with blood/body fluids
- after removing gowns/coats and gloves
- before eating/drinking and leaving the laboratory
- Ideally, liquid soap dispensers should be provided to the laboratories, which should be regularly cleaned and maintained. If not feasible, soap bars after washing should be left in a dry tray to prevent contamination with some microorganisms which grow in moist conditions
- A moisturising hand cream should be used after every hand wash
- Gloves should not be regarded as substitute for hand washing

iii) Safe techniques

- Use of biological safety cabinets – Biological safety cabinets Class 2 should be used for handling materials containing higher concentration of infectious agent than usually present in clinical specimens e.g. while carrying out HIV culture.
- All procedures and manipulations of potentially infectious material should be performed carefully to minimise the formation of droplets, aerosols, splashes or spills.
- Mouth pipetting should be strictly prohibited. Mechanical pipetting devices should be used for pipetting of all liquids in the laboratory.
- Centrifugation should be done in tubes with safety caps.

iv) Safe Handling of sharps

- Extreme care should be used to avoid auto-inoculation.
- All chipped or cracked glassware should be discarded in appropriate containers.
- Broken glass should be picked up with a brush and pan. Hands must never be used.
- The disposable needles should never be manipulated, bent, broken, recapped or removed from syringes.
- The used sharps should never be passed directly from one person to another.
- Always one should dispose of his/her own sharps.
- Used needles should be discarded in puncture-proof rigid containers (plastic or cardboard boxes) after disinfection in 0.5-1% freshly prepared sodium hypochlorite solution (common bleach) and never in other waste containers. If a needle shredder is available, only the needles or the needles along with syringe nozzle may be shredded depending upon the type of the shredder
- Sharp disposable containers should be located close to the point of use.
- Sharp disposal containers should be sent for disposal when three-fourth full.

v) Safe handling of specimens (blood etc.)

- Specimens, specially blood and body fluids should be collected in pre-sterilised screw-capped plastic containers properly sealed to prevent spillage or leakage.
- Autoclaved/pre-sterilised disposable syringes and needles for venepuncture or lancets/ cutting needles for finger prick should be used.
- Cuts in hands should be properly covered with waterproof adhesive bandages.
- Disposable gloves should be worn while collecting blood/body fluids and proper asepsis should be maintained.
- If a sample shows evidence of breakage (in case not collected in the above container), leakage or soiling, it should be transferred with a gloved hand into a second sterile container. Any pertinent information should be rewritten from the old to the new container.
- If the requisition slip is contaminated with blood, it should be rejected. In case of emergency, the contaminated slip may be handled using gloves.
- Hands should be thoroughly washed with soap and water particularly after handling specimens.
- Blood and other specimen containers should be labelled with a special warning sign "Biohazard precaution". If the outside of the container is visibly contaminated with blood it should be cleaned with disinfectant. All blood specimens should be placed in small leak-proof impervious plastic tubes for transportation to the laboratory.

vi) Safe Handling of blood/body fluid spills

In case of a spill of blood/body fluid in the laboratory, the area should be flooded with a disinfectant solution. e.g. freshly prepared 0.5-1% Sodium hypochlorite solution and left for 30 minutes. After wearing gloves, the area should be covered with paper towels or gauze sponges to absorb the liquid followed by a thorough wash with soap and water. All contaminated materials should be disposed of as infectious waste.

Hepatitis B Vaccine

Appropriate use of HBV vaccine plays a key role in prevention of transmission of HBV from patient to health care worker. Therefore it is important that all the HCW's including the laboratory workers should be immunized as recommended by WHO.

Laundry and linen

Although soiled linen has been identified as a source of large numbers of certain pathogenic organisms, the risk of actual disease transmission is negligible.

Soiled linen may be handled as little as possible and with minimum agitation to prevent gross microbial contamination of the air and of persons handling the linen.

All soiled linen must be handled with gloved hands and if feasible, decontaminated in 0.5-1% sodium hypochlorite in the laboratory before sending to the laundry.

Soiled linen after decontamination, should be put in heavy plastic bags which are tied and sent to the laundry. In the laundry, decontamination in bleach is recommended in case

not done earlier. This should be followed by washing in hot water (>70°C) with detergent. Usual wash cycles in washing machines kill HIV.

Management of Accidental Exposure to HIV in the Laboratory:

Accidental exposure and post-exposure prophylaxis is discussed in Chapter 18.

General bio-safety guidelines for laboratory workers

i) Access to laboratory should be limited at the discretion of laboratory- in-charge when experiments are in progress. The door should have a symbol of BIOHAZARD; NO ADMITTANCE. Laboratories should be well ventilated so as to ensure that the personnel do not breathe in contaminated air.

ii) Eating, drinking, smoking and application of cosmetics are prohibited in the laboratory. Sandals and open style shoes do not afford proper foot protection and are not to be used. Contact lenses especially the soft ones should not be worn.

iii) Laboratory and work tables should be scrupulously cleaned with liquid detergents and disinfectants. Laboratory work surface should be decontaminated once a day after completion of day's activity and immediately after spill of viable material with disinfectant.

iv) Biological safety cabinets and other primary containment devices e.g. centrifuge safety caps should be used whenever handling hazardous specimens and when it is likely to produce aerosols or infectious droplets.

v) Blood and other specimen containers should be labelled with a warning sign. The outside of the specimen container should be cleaned with sodium hypochlorite solution in case of visible contamination.

vi) Gloves should be worn while dealing with blood specimens, blood-soiled items, body fluids, excretions, secretions, surface materials and objects exposed to them.

vii) Gowns/laboratory coats must be worn while working with potentially infective materials and removed before leaving the laboratory.

viii) Hands should be washed immediately after contact with blood and before leaving the laboratory.

ix) Mechanical pipetting devices should be used. Mouth pipetting is strictly prohibited. Careful techniques should be followed to minimise the creation of aerosols.

x) Accidental wounds from sharp instruments should be avoided. Extreme caution is warranted when handling needles and sharps to avoid auto-inoculation. Needles should be promptly placed in a puncture resistant container immediately after decontamination.

xi) Paper work should not be done on potentially contaminated surface.

xii) All potentially contaminated materials and wastes from the laboratory should be disposed after decontamination preferably by autoclaving. A label with a globally accepted biological hazard sign should be applied.

4.3.2 Effective use of sterilization and disinfection

4.3.2.1 Definitions

- **Cleaning** is a process which removes foreign material (e.g. soil, organic material, micro-organisms) from an object.
- **Disinfection** is a process which reduces the number of pathogenic micro-organisms, but not necessarily bacterial spores from inanimate objects or skin, to a level which is not harmful to health.
- **High level disinfection** is often used for a process which kills mycobacterium tuberculosis and enteroviruses in addition to other vegetative bacteria, fungi and more sensitive viruses.
- **Sterilization** is a process which destroys all micro-organisms including bacterial spores.

  The level of decontamination should be such that there is no risk for infection when using the equipment.

4.3.2.2 Classification of infection risk from equipment or environment into three categories and suggested levels of decontamination.

- **Low risk:** Items in contact with normal and intact skin, or the inanimate environment not in contact with the patient (e.g. walls, floors, ceilings, furniture, sinks and drains). Cleaning and drying is usually adequate except when there is spill of blood/body fluids.
- **Intermediate risk:** Equipment which does not penetrate the skin or enter sterile areas of the body but is in contact with mucous membranes or non-intact skin, or other items contaminated with virulent or transmissible organisms (e.g. respiratory equipment, gastro-intestinal endoscopes, vaginal instruments, thermometers). High level disinfection is usually adequate.
- **High risk:** Items penetrating sterile tissues, including body cavities and the vascular system (e.g. surgical instruments, intra-uterine devices, vascular catheters, syringes and needles etc.). Decontamination followed by cleaning and sterilisation is required. High level disinfection may sometimes be appropriate if sterilisation is not possible.

4.3.2.3 Methods

Cleaning of glasswares:

- Thorough cleaning and drying with detergents and water remove most organisms from an object/surface and should be carried out meticulously before sterilization.
- It may be manual or mechanical (ultrasonic cleaners or washers/disinfectors) Manual – All contaminated items after prior disinfection should be dismantled before cleaning. Cold water is preferred as it will remove most of the protein materials (blood, sputum, etc.). The most simple, cost effective method is to thoroughly brush the item keeping the brush below the surface of the water to prevent the release of aerosols. The brush should be decontaminated after use and dried. The items should be rinsed finally in

clean, warm water and dried. Items are then ready for use for sterilisation. Personnel handling contaminated items should wear good quality gloves for personal protection.

Environmental cleaning:

- Floors, surfaces, sinks and drains should be cleaned with warm water and detergent. Routine use of disinfectants is unnecessary.
- If there is spillage of blood, body fluids or sputum, disinfection before cleaning is recommended. In high risk areas or following spillage from a known infected patient, the surface is cleaned using freshly prepared 0.5-1% sodium hypochlorite solution as described under 4.3.1.3. Gloves should be worn. Release of chlorine gas from disinfection of large spillage can be hazardous to staff. If spillage is immediately removed, general disinfection of the room is not necessary. Thorough cleaning will suffice.

Disinfection: Most of the disinfectants used in health care settings in this country, if used in proper concentration and for suitable period of time are effective against HIV. Either thermal or chemical processes are used for disinfection. Thermal disinfection is preferred whenever possible. It is generally more reliable, leaves no residue, is more easily controlled and is non-toxic. Organic matter (serum, blood, pus or faecal matter) interferes with the antimicrobial efficiency of either method. The larger the number of microbes present, the longer it takes to disinfect. Boiling (100°C) for 20-30 minutes (holding time) is a very simple and reliable method for the inactivation of all micro-organisms including hepatitis B virus, HIV and mycobacteria. Carefully done, it is a high-level disinfection procedure. The main use of chemical disinfectants is for heat labile equipments where single use is not cost effective. The properties of common chemical disinfectants are shown in **Table 4.2**.

Table 4.2 Disinfectant properties of commonly used disinfectants.

	Anti microbial activity					Other properties			
	Spore	Myco bacteria	Other bacteria	Virus		Stable	Inactivation Org. matter	Corrosive/ damaging	Irritant/ sensitising
				Env.	N.Env.				
1. Alcohol (70%)**** (1-10 min.)	None	Good	Good	Good	Moderate	Yes in closed container)	Yes (Fixative)***	Slight (Len cement)	No
2. Chlorin rel. cmpds. (0.5-1%) (10-60 min.)*****	Good	Good	Good	Good	Good	No (< 1 day)	Yes	Yes	Yes*****
3. Glutaraldehyde 2% (5 min - 3 hrs)*****	Good (3 hours)	Good* (20 min.)	Good (5-10 min)	Good (5-10 min)	Good (5-10 min)	Moderate (14-28 days)	No (Fixative***)	No	Yes***
4. Clear soluble phenolic (1-2%)**	None	Good to moderate	Good	Moderate	Poor	Yes	**No	Slight	Yes

* Less active against M.avium intracellulare
**Potentially toxic, not to be used in neonatal ward
***Poor penetration
****Flammable
***** Irritant/sensitizing in high concentration

E-enveloped
NE-non enveloped

Decontamination/disinfection of used needles and syringes:

- The needle is not detached from syringe.
- The disinfectant is aspirated into the syringe.
- The needles and syringes are immersed with disinfectant horizontally in a flat tray for 30 minutes (**Fig. 4.3**).
- The disinfectant solution is discharged from the syringe and needle and rinsed with disinfectant by filling and emptying a number of times.
- The disposable syringes and needles are disposed as in 4.3.3.
- The reusable syringes and needles are autoclaved/boiled for 30 minutes.
- Chemical disinfection should never be used as a method of sterilization particularly for syringes and needles.

Sterilisation:

- Sterilisation is carried out by steam under pressure, dry heat, gas or liquid chemicals.
- The choice of the methods like autoclaving, use of hot air oven etc. depends on a number of factors including type of material of the object, number and types of organisms involved and risk of infection to patients or staff.
- HIV is a fragile virus and is adequately inactivated by standard sterilisation procedures like autoclaving at 15 pounds pressure for 20 minutes at 121°C or hot air oven at 160°-180°C for 1 hour (holding time).
- Pressure cooker (household pressure cooker or WHO/UNICEF modification) achieves 15 p.s.i. equivalent to 121°C for 20-30 minutes and may be used in small laboratories.
- Any sterilization procedure should be monitored routinely by mechanical, chemical and biological techniques.
- Sterile items should be protected against recontamination.

4.3.3 Safe disposal of laboratory wastes

Laboratory wastes are potential hazards. Infectious waste can transmit numerous diseases in the community and put those who handle waste and live on it's proximity, at risk. Besides, the increasing use of disposables in health care is also posing an additional burden on the waste management facility. It is extremely important that the recycling of these items is prevented. Only a small percentage (<10%) of the waste generated in health care settings are infectious while another 5% is non-infectious but hazardous. The most practical approach to the management of biomedical waste is to identify and segregate infectious waste (with a potential for causing infection during handling and disposal), for which some special precautions appear prudent. This will drastically reduce the cost of the disposal methods in health care settings.

4.3.3.1 Setting up of biomedical waste facility

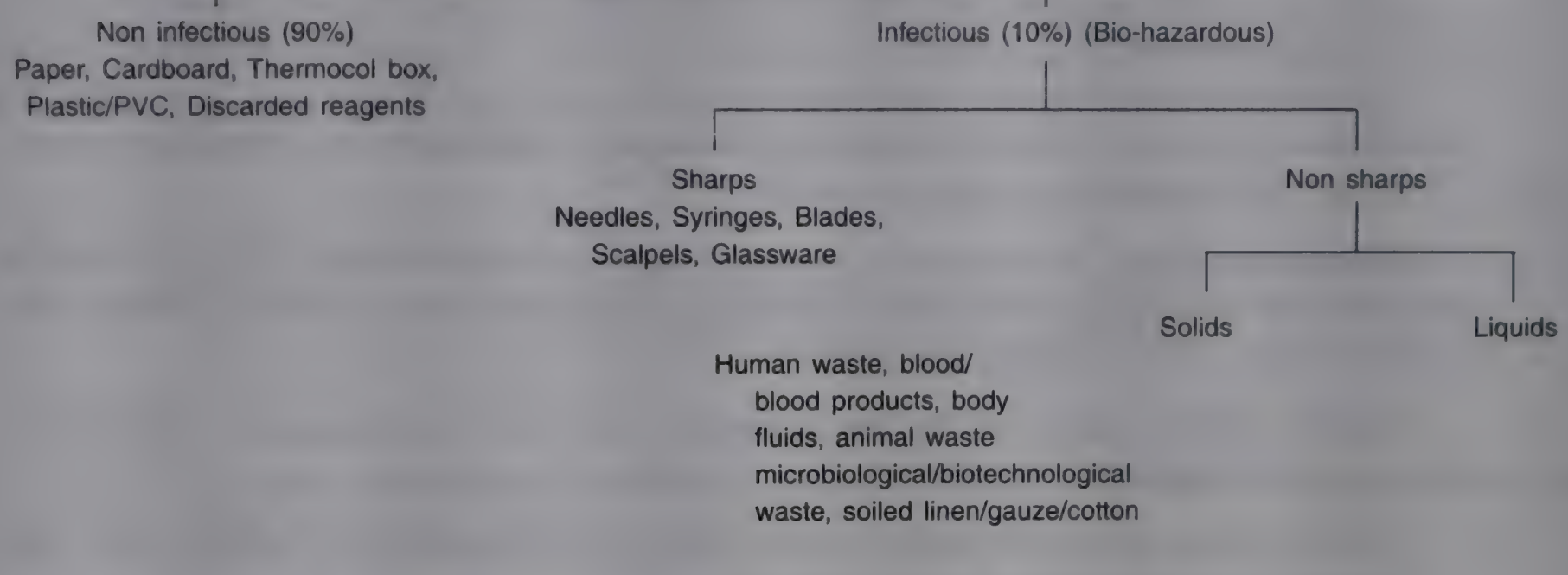
Every hospital, nursing home, veterinary institution, animal-house, blood banks, research institutes generating biomedical wastes should install an appropriate biomedical waste facility

in the premises or should set up a common facility in accordance with the directions given by the appropriate authority. Biomedical waste should not be generated without authorisation. Every hospital should have a waste management programme Waste survey is an important part of the waste management programme and helps in determining both the type and quantity of waste being generated in the hospital including the laboratory and determine the feasible methods of disposal.

4.3.3.2 Classification of laboratory wastes:

Wastes generated in the laboratory are classified in **Table 4.3**.

Table 4.3 Laboratory waste



4.3.3.3 Containing waste at generation point

At the generation point i.e. the laboratory, waste is managed in the following way:

- Collection
- Segregation and weighing
- Storage

Waste segregation is the key to any waste management scheme. It consists of placing different types of waste in different containers or colour-coded-bags at the site of generation. This helps in reducing the bulk of infectious waste and contains spread of infection to general waste. This practice reduces the total treatment cost, the impact of waste in the community and the risk of infecting workers. Proper segregation should identify waste according to source and type of disposal/disinfection.

Waste should be segregated into different categories (**Table 4.3**) at the site of generation i.e. in the laboratory and weighed separately at the time the waste is being disposed.

Solid Non infectious waste is collected in black bags and disposed as household waste.

Infectious waste must be separated at the point of generation itself and should be decontaminated prior to it's storage, transport and disposal. Solid infectious waste are disposed as follows:

- Sharps a) Needles and syringe nozzle-shredded in needle-destroyer (if available); if not available decontaminated as in b).
- b) Scalpel blades/lancets/broken glass-should be put in separate container with bleach, transferred to plastic/cardboard boxes, sealed to prevent spillage and transported to incinerator.
- Glass wares should be disinfected, cleaned and sterilised.
- Culture plates with viable culture should be autoclaved in plastic autoclavable bags, media are removed and collected in yellow bags and disposed of by incineration/ micro-waving/hot air oven. The plates can be reused after sterilization.
- Swabs should be chemically disinfected followed by incineration.
- Disposable items include single use products (syringes, gloves, sharps etc.)- As these items are often recycled and have the risk of being reused illegally, these should be disinfected by dipping in freshly prepared 1% Sodium hypochlorite for 30 minutes to 1 hour. Bins which can be used for this purpose are a set of twin bins, one inside the other with the inner one being perforated and easily extractable. This minimizes contact when the contents are being removed.

Disposable items like to gloves, syringes etc. should be shredded cut or mutilated before disposal followed by deep burial or properly accounted before disposal. Extreme care should be taken while handling the needles.

Liquid wastes generated by the laboratory are either pathological or chemical in nature and are disposed of as follows:

- Non-infectious chemical waste should first be neutralised with reagents and then flushed into conventional sewer system.
- The liquid infectious waste should be treated with a chemical disinfectant for decontamination then neutralized and flushed into the sewer.

Collection bags: Solid wastes are collected in leak-resistant single heavy duty bags or double bags may be used. Bags having different colour codes (**Table 4.4**) with red labels mentioning date and details of waste are recommended. The bags are tied tightly after they are three-fourths full.

Packing, storage and transport

- All segregated and disinfected waste should be packed in proper containers and colour-coded bags (**Table 4.4**) with red labels mentioning details of biomedical waste and biohazard signs (**Fig. 4.4**). All containers used for storage of such waste shall be provided with a properly covered lid.
- Such containers should be inaccessible to scavengers and protected against insects, birds, animals and rain.
- There should not be any spillage during handling and transit of such waste.
- The waste sharps, after pre-treatment should be broken before packing in the container.
- The waste should be transported in vehicles authorised for this purpose only.

Table 4.4 Container and colour coding for disposal of bio-medical wastes

Waste category	Waste class	Type of container	Colour coding	Treatment/Disposal option
No. 1	Human anatomical waste, blood & body fluids	Single-use containers/ Plastic holding bags	Red	Incineration/Deep Burial
No. 2	Animal & slaughter house waste	Single-use containers/ Plastic holding bags/sacs	Orange	Disinfection & Deep Burial
No. 3	Microbiology & bio-technology waste	Single-use containers/ Plastic holding bags	Yellow	Autoclaving/Microwaving & Incineration
No. 4	Waste sharps	Re-usable/single-use sturdy containers of plastic, glass or metal	Blue	Shredding & Deep Burial
No. 5	Discarded medicines	Re-usable/sturdy cardboard/ glass/plastic holding bags	Blue	Shredding & Deep Burial
No. 6	Soiled wastes (linen)	Plastic bags/sacs	Yellow/ Black	Disinfection and machine cleaning
No. 7	Disposables (other than sharps)	Re-usable/sturdy containers/ plastic holding bags	Yellow/ Black	Disinfection-Chemical/ Autoclaving, Shredding, Burial
No. 8	Liquid wastes	NA	NA	
No. 9	Chemical wastes	Sturdy containers/plastic holding bags	Yellow/ Black	

- No such waste should be stored in the place where it is generated for a period of more than two days.

4.3.3.4 Treatment and disposal

Disposal methods: Disposal may be done by:

- Municipal corporation
- Sanitary landfill
- If incinerator is not available, deep burial in controlled landfill sites is recommended. Decontamination should be carried out before burial.
- Incineration (Temp. 750°C)

  Incinerator-burns/reduces the infectious waste to ashes and therefore favoured by hospitals. It may be of two types – common or individual. There are some disadvantages like pollution/incomplete melting of needles. Hospitals with more than 30 beds or >1000 patients per month should have an incinerator. Plastics cannot be incinerated.

4.3.3.5 Guidelines for waste disposal

- All biomedical wastes should be treated and disposed of strictly in accordance with the options mentioned in the **table 4.4**.
- Waste which cannot be incinerated, (plastics) should be pre-treated by disinfection and disposed of in an environmentally sound manner.

- Waste should not be dumped, discharged or disposed in any place other than a site identified for the purpose.
- All precautions and personal safety measures should be taken (including provision of protective clothing, masks, gloves, gumboots, goggles, etc. as may be necessary). Hepatitis B vaccine is recommended for affording protection to all personnel engaged in handling biomedical waste, or being exposed to such wastes against infection from handling or exposure.
- All treatment and disposal facilities should be located at a specified area away from the general service area of the hospital, public places and residential areas.
- When the treatment option is burial, the pits should be located at sites away from agricultural land, residential areas, ground-and safe water sources. There should be no leakage from the pits in to surrounding areas.
- All plastics should be disinfected, shredded and disposed of in an environmentally friendly manner. Recycling of disposables e.g. syringes, needles, gloves, transfusion bags etc. should be prevented.
- All liquid waste should be disinfected and flushed in the sinks at the point of generation.
- Biomedical waste should not be disposed of on open land and municipal dustbins. Untreated liquid waste should not be let into sewers.

4.3.3.6 Maintenance of records

Separate records for classification of waste and their regular disposal should be maintained in the laboratory. The waste disposal programme should be supervised and monitored regularly.

4.3.3.7 Reporting of accidents

In the event of an accident occurring at any location or site where biomedical wastes are handled or during transportation, the appropriate authorities must be informed and needful action taken.

4.4 TRAINING

Training regarding need of and national guidelines of bio-safety practices is extremely important and should be provided at regular intervals for different levels of health care workers. Guidelines for bio-safety should be provided which may be modified from time to time according to requirement.

4.5 IMPLEMENTATION OF BIO-SAFETY PRACTICES IN LABORATORY

Although guidelines regarding universal precautions and other bio-safety practices are available since long, strict implementation is not in practice in healthcare settings in India even in the capital city. With increase in the prevalence of HIV infection, there is a definite need that the HCW's take bio-safety practices seriously. For effective compliance, the laboratory managers should ensure adequate supply of personal protective equipments, availability of

materials for handwashing, disinfectants and set up an effective waste disposal programme for disposal of biomedical wastes.

4.6 SOURCE OF LITERATURE AND SUGGESTED FURTHER READINGS

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- Ministry of Environment and Forests notification New Delhi the 16th October, 1997.
- Shrishti: Implementing Hospital Waste Management: A guide for health care facilities. September, 1996.
- WHO Laboratory Bio-safety Manual 2nd Edition, 1993 World Health Organisation, A.I.T.B.S. Publishers and Distributors.
- WHO Managing Medical Waste in Developing Countries, 1994 World Health Organisation, Geneva.
- AIDS in India. News letter of the National AIDS Control Organisation, September 1993.
- Perspectives in Disease Prevention and Health Promotion update Universal Precautions for prevention of transmission of Human Immunodeficiency Virus, Hepatitis B Virus and other Blood-borne Pathogens in Health-care Settings. MMWR 37(24), 377-388.
- Centers for Disease Control. Recommendations for prevention of HIV transmission in health-care settings. MMWR 1987: 36 (suppl no. 2S).

Chapter 5

COLLECTION AND TRANSPORT OF SPECIMENS FOR HIV TESTING

Almost all the laboratory procedures for HIV testing are performed on patients' blood; serum or plasma; hence the collection of blood is described below.

5.1 PERFORMING VENEPUNCTURE

- Gloves should be worn and sterilised/disposable syringes and needles should be used.
- For avoiding soiling, a piece of linen with a layer of dressing pad (a sheet of absorbent cotton between two layers of gauze piece) or simply a big piece of absorbent cotton may be placed below the forearm before commencing venepuncture.
- After collecting 5 ml of blood aseptically, it should be carefully transferred from the syringe without squirting in to a sterile plastic leak proof specimen container preferably screw capped. The containers should be labelled before commencement of venepuncture. If the vial has anticoagulants, then a second person wearing gloves would have to help in shaking the vial for mixing the blood well with the anticoagulants. The cap may be tightly screwed after the blood has been transferred to the vial.
- After blood is collected, the tourniquet is removed and the needle is withdrawn. The patient is given a dry sterile cotton swab to press over the site of venepuncture. Elbow may be flexed to keep the cotton swab in place till the blood stops. Any blood spill is carefully wiped with 70% ethanol.
- All the swabs and cotton pieces are placed in plastic bags for disposal. If the outside of the vial is visibly contaminated with blood, it should be cleaned with 10% freshly prepared sodium hypochlorite solution.
- The blood is allowed to clot for 30 minutes (not more than 2 hours) at room temperature. The clot may be gently broken if necessary using sterile pasteur pipettes.

5.2 SEPARATION OF SERA SAMPLES

- The vial is centrifuged at 1200 g (3000 rpm) for 10 minutes to separate serum to avoid haemolysis. If no centrifuge is available, the blood with clot may be left in the refrigerator at +4°C for overnight. The clot will retract and get separated from serum.
- The specimen vial is un-stoppered, the serum is drawn off by sterile pasteur pipette and transferred to a sterile plastic screw capped leak proof tube.

5.3 ADDITION OF PRESERVATIVE

- Preservatives should not be added since it inactivates conjugates and gives rise to false serological results.

- If necessary, 5 bromo, 5 nitro, 1-3 dioxane in propylene glycol at a final concentration of 0.05% is recommended as preservative (chapter 10 and 11).
- Thiomersal at a final concentration of 0.01% is effective only for a few weeks as it loses activity when exposed to light.

5.4 STORAGE OF SERUM SPECIMENS

- The sera samples are placed in leak proof plastic containers in the refrigerator at +4°C, for storage.
- The outside of the container is checked for visible contamination with blood which should be cleaned as in 5.1.
- Then the specimen vials are packed in a second tightly capped unbreakable container surrounded by adequate packing material.
  For storage for a long time, deep-freezing at –70°C is advised.

5.5. TRANSPORT OF SERA SAMPLES

- The specimen tube in which serum is to be transported, should not have crack or leakage. Preferably, it should be made of plastic and should be screw capped. The outside of the container is checked for any visible contamination with blood which should be disinfected as in 5.1.
- The tube is labelled and then placed in a second tightly capped unbreakable container (**Fig. 5.1**) surrounded by adequate packing material (like tissue paper, adsorbent cotton etc.) to absorb liquid, if leakage occurs accidentally.

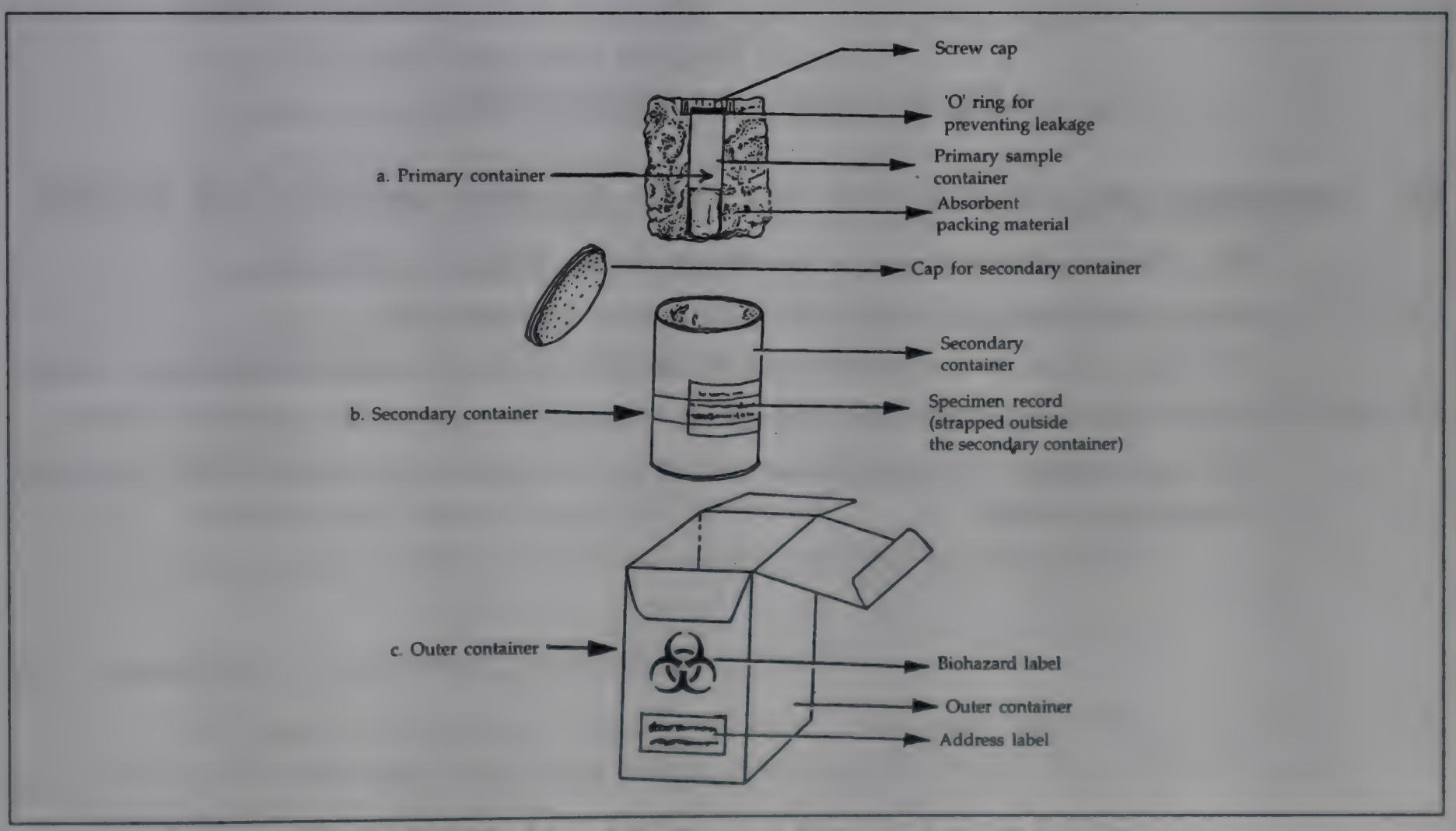


Fig. 5.1 Packing infectious substances for the post

- The secondary container should also have a label. This is placed in a thermocol box having ice packs to maintain proper cold chain system during transit.
- A proforma with details i.e. name, age, sex, risk factors, history of previous testing etc. should accompany the specimen.
- A biohazard symbol (**Fig. 5.2**) must be fixed outside the thermocol box. This box can now be sent to a distant laboratory.

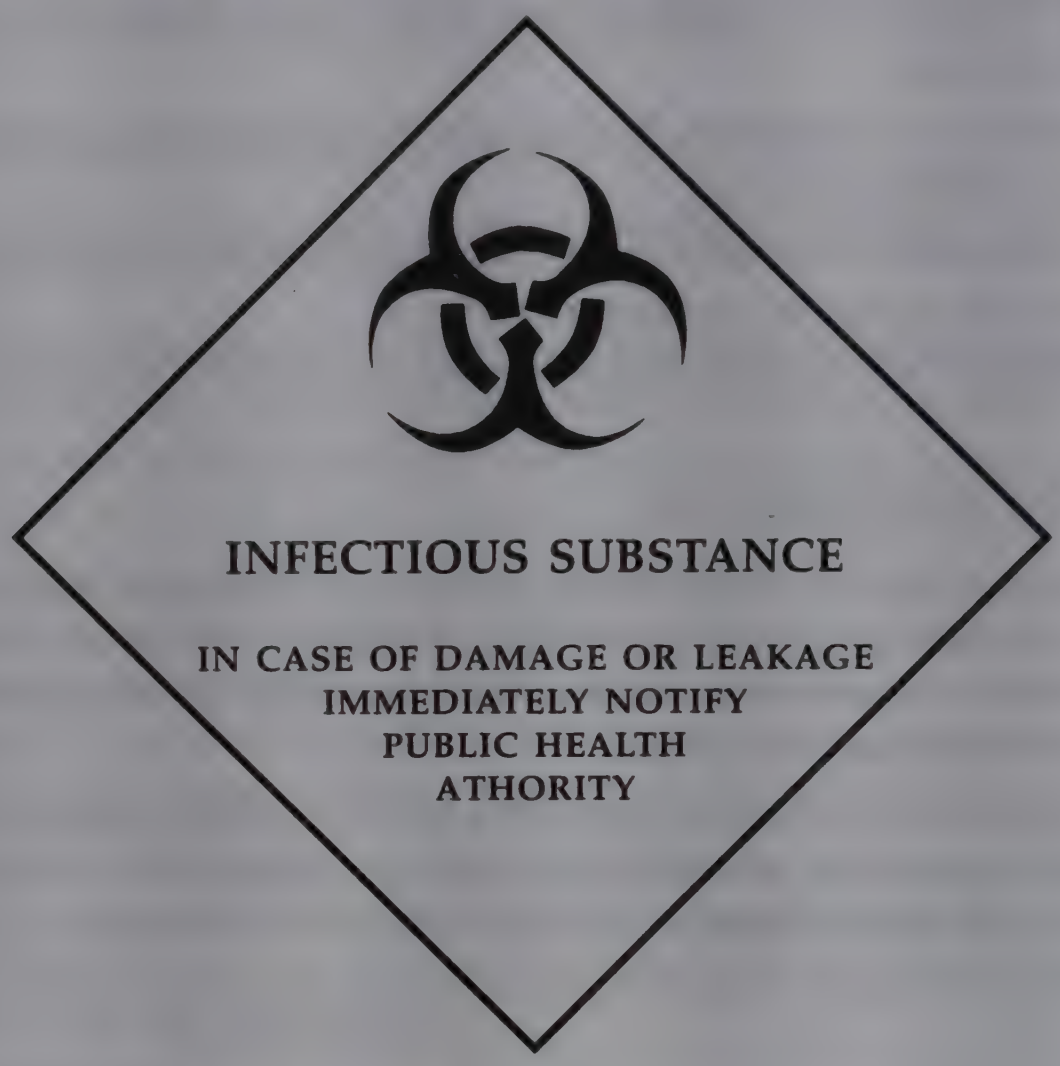


Fig. 5.2 Infectious substance label

5.6. TRANSPORT OF WHOLE BLOOD FOR VIRUS ISOLATION AND CD4, CD8 STUDIES

- The universal precautions as mentioned above should be followed.
- Blood is collected in heparinized/EDTA screw capped vials.
- It is then placed in a second unbreakable container with adequate packing material.
- Should be transported in wet ice so as to reach the laboratory within 24 hours.
- For virus isolation, if the specimen cannot be transported immediately after collection, it should be frozen.

Chapter 6

HIV ANTIBODY TESTING WITH SPECIAL REFERENCE TO HIV-1

6.1 INTRODUCTION

HIV/AIDS is not like other infectious diseases. It is far more complex because HIV infection is life long, outcome is invariably fatal and no cure or vaccine is available so far. Since, commonly HIV/AIDS is acquired through sexual contact, individuals known to be HIV infected are stigmatized and discriminated against. A number of moral, ethical, legal and psychosocial issues are related with a positive HIV status. So, anyone attempting to assess the HIV status of an individual must be conversant with these issues, strategies of HIV testing, protocols of testing, rationale of using test kits, correct method of informing the client, counselling, importance of confidentiality, technical and other pitfalls and quality assurance to name some. Confidentiality of a positive test result is of utmost importance. Counselling should be undertaken to motivate the individual to tell the spouse/family and induce behaviour change.

Another important point to understand is that laboratory diagnosis is the only method determining the HIV infection status of an individual during the long asymptomatic period.

6.2 PURPOSE OF HIV TESTING

- Information is useful for prophylaxis, medical management and treatment of HIV and related illnesses.
- To assure blood safety and donation safety.
- To assess the efficacy of targeted intervention in a defined cohort.
- To monitor trends of epidemic (sentinel surveillance etc.).
- Identification of asymptomatic individuals (practising high risk behaviour).
- To plan personal and family's future if the result is positive.
- To motivate for behaviour modification through counselling amongst those who test negative and who practise high risk behaviours.
- To induce behaviour change and prevent transmission by counselling in those who test positive.
- To diagnose clinically suspected cases.
- For peace of mind of individuals practising high risk behaviour.

6.3 KINETICS OF HUMORAL IMMUNE RESPONSE

An understanding of the sequence of events that follow the entry of virus into the body will help to understand the optimal usage of various HIV tests during different stages of HIV disease.

Viral entry into the body leads to a transient period of plasma viraemia (usually high level) and p24 antigenaemia. However, the levels of these components come down with concomitant immune response. Humoral response is evidenced by formation of antibodies of different classes against different structural proteins (gag: p15, p17, p24, p55; env: gp 41, gp 120, gp 160; and pol: p31, p51 and p66), regulatory proteins (nef, rev, tat) and accessory proteins (vif and vpu). All structural components are strongly immunogenic and induce formation of antibodies, whereas immunogenicity of regulatory and accessory proteins is variable.

The antibodies appear in blood within 2-8 wks after infection but usually become detectable after 3 wks to 12 wks. with the assays available presently. This period following the entry of HIV into the body and the appearance of detectable levels of antibodies with the available test kits is called the **"window period"**. During this period the individual is infected, infectious and non-reactive with the antibody detection tests. The antibodies to gag protein (p24 and p55) are first to appear usually, though antibodies to env proteins and pol proteins may also be produced simultaneously. As infection progress to AIDS antibody to p24 usually declines as p24 antigen levels rise concomitant with progression of disease to AIDS. However, antibodies to env proteins persist throughout the infection (**Fig. 6.1**).

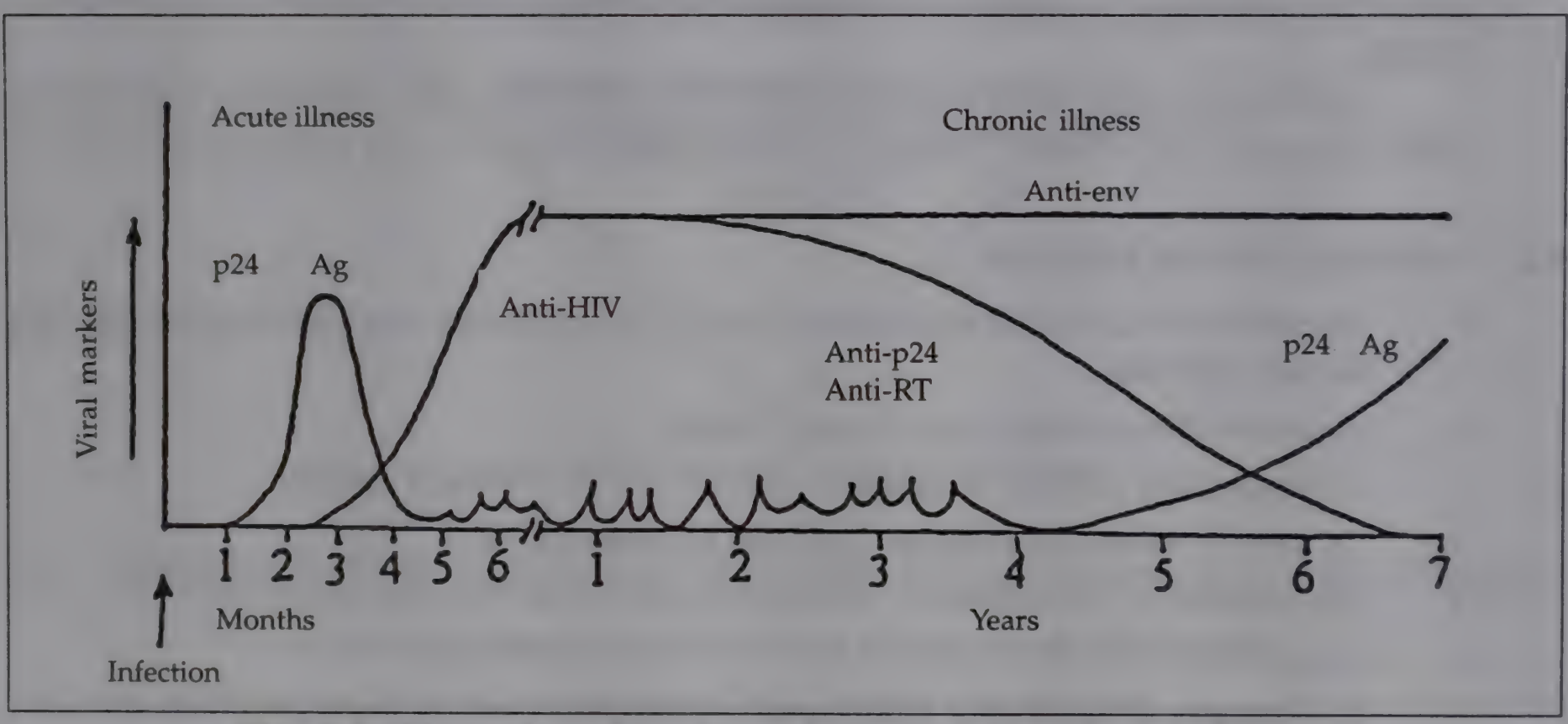


Fig. 6.1 HIV markers from time of infection to endstage disease

Anti-HIV antibodies may be IgA, IgM and IgG. The IgA and IgM responses are inconsistent. IgG response is consistent and better understood. IgM response appears earlier than IgG but the sensitivity of assays available to detect this class of immunoglobulins is low and also IgM is detectable for a short period. Detection of IgM is valuable for identifying early seroconversion particularly following needle stick injury (2-11 days) and infection in newborn. IgA is the predominant immunoglobulin in seromucous secretions (saliva, colostrum, genito-urinary secretions etc.). Since IgA does not cross placenta and therefore detection of anti-HIV IgA by HIV-IgA assay in a newborn is diagnostic of congenital HIV infection. However, Enzyme Linked Immunosorbent Assay (ELISA) and Western Blot (WB) to detect IgA and IgM need further evaluation.

6.4 INFORMED CONSENT AFTER PRETEST COUNSELLING (See chapter 15 for counselling for HIV testing)

HIV testing for the purpose of identification of an individual must always be undertaken after pre test counselling and informed consent. Testing without explicit consent has proven to be counter productive and has driven the HIV positive individuals underground. This makes institution of prevention and intervention measures more difficult. Pretest counselling also prepares the individual to face the HIV test result.

6.5 CONFIDENTIALITY

The confidentiality of the test result (both negative as well as positive) should be strictly maintained in most cases. This is to respect the privacy and rights of the individuals and to protect them from discrimination, victimization and stigmatization. The test result, name of the individual etc. must never be discussed loosely. The test report must be placed in a sealed envelope and submitted to the clinician who requisitioned the test. The envelope should be marked "confidential". The records in the laboratory must also be kept secure to prevent access by unauthorised persons. As far as possible the results never be communicated via telephones.

6.6 DETECTION OF HIV SPECIFIC ANTIBODIES

Detection of anti-HIV antibodies is the mainstay of testing for HIV and diagnosis of HIV.

Tests to detect specific HIV antibodies can be classified into:

- Screening tests (ELISA/EIA, Rapid, Simple)
- Supplemental tests

Screening tests are performed to screen units of donated blood and blood products and for surveillance. These include a myriad of ELISAs which usually take 2-3 hrs to yield results. Rapid screening tests give results within minutes and include visual assays like dot-blot tests, particles (gelatin, RBC, latex, microbeads) agglutination, HIV spot and comb test and fluorometric microparticle technologies. Simple screening tests are also based on ELISA principle but take about half an hour or so. As far as sensitivity and specificity of serening tests, approved by NACO are concerned it should be 99.7% and 95% or more.

Supplemental tests are performed on serum sample reactive in screening test for the purpose of diagnosis and identification of the individual. When a serum specimen is reactive by any one of the screening tests it has to be tested again by a different system using different HIV antigens or different principle of test to confirm the diagnosis. If a specimen is reactive in 2 different systems it has to be tested again using one of the supplemental tests which may be a third ELISA/Rapid/Simple test or a Western Blot test (WB) as the case may be.

A healthy individual reactive in three different systems of testing is confirmed to be having HIV infection. The other supplemental tests like W.B./IF are used to resolve discordant results of ELISA and research. As far as India is concerned as these tests are expensive, time consuming and need expertise.

Whichever commercial kit is selected it should be ensured that it detects antibodies against both HIV-1, HIV-2 and their subtypes.

Presence of HIV antibodies indicates the individual is infected with HIV and can transmit infection to others through unsafe risk behaviour.

6.7 SPECIMENS TO BE COLLECTED FOR DETECTION OF ANTI-HIV ANTIBODIES

Most common specimen is blood. HIV test kits are available for detecting HIV antibodies in various kinds of specimens like blood, plasma, serum, saliva and urine.

Antibody detection:

- Blood/serum/plasma
- Saliva ↕ not as good
- Urine ↕ as blood

6.8 HIV ANTIBODY SCREENING ASSAYS

The list of the screening assays for HIV testing is given below

- ELISA (2-3 hours)
- Rapid tests (Minutes)
  - Dot blot assays
  - Particle agglutination
  - HIV spot and comb tests
  - Fluorimetric microparticle technologies
- Simple: Based on ELISA principle

  Take ½ an hour

ELISA is the most commonly performed screening test. Screening assays must detect all positive sera i.e. should be highly sensitive even if some false positives do occur. However, results of a screening test should never be used as the final interpretation of HIV status, and individual is never identified on the basis of one screening assay as technical errors can occur. The serum reactive in screening assay is subjected to confirmatory tests (as per policy and strategy of testing) to be classified as reactive only if it is reactive in repeated assays.

Other screening tests were also introduced subsequently. These include latex, red cell and gelatin particle agglutination, comb tests and dot- blot assays. These tests are easy to perform, are rapid, do not require sophisticated equipment, technical expertise and are mostly cost effective. Some of them, particularly comb tests and dot- blot assays, are also discriminatory for HIV 1 and HIV 2 antibodies.

6.9 ELISA AS THE SCREENING ASSAY

ELISA is the most commonly performed screening test. It is easy to perform, adaptable to large number of samples, is sensitive and specific and cost effective. Some of the assays

take 5 minutes and others as long as 22 hrs; some need only 1 µl serum, others may need as much as 800 µl ; some need sophisticated instruments, others need none; some are easy to perform and others need expertise and are difficult to perform; some are expensive and some are not so expensive, so the appropriate test choice can be made taking into consideration the available resource, storage facility, technical expertise available, infrastructure available, objective of testing, prevalence of infection and performance characteristics of test kits etc.

6.9.1 Different types of HIV kits (based on type of HIV antigen) available commercially

First generation of ELISA developed were very sensitive but not specific because whole viral lysates were used as antigen. These lysates usually contained small amounts of host cell components which gave rise to false positive reactions. The ELISA technologies were improved and 2nd and 3rd generation kits were developed using recombinant and synthetic peptides as antigens. So, ELISA assays available in the market may be:

- First generation: use antigens derived from detergent disruption of viruses grown in human lymphocytes.
- Second generation: use artificially derived recombinant antigens expressed from bacteria or fungi.
- Third generation: use chemically synthesized oligopeptides of about 15-40 amino acids (synthetic peptides).

6.9.2 Principles of ELISA

On the basis of the principle of the test ELISA can be divided into:

- Indirect
- Competitive
- Sandwich
- Capture assays

All ELISA consist of either HIV antigen or antibody (depending upon the principle) attached on a solid phase (matrix or support) and, incorporate a conjugate and substrate detection system. Viral antigens may be whole virus lysates, recombinant or synthetic peptides. The matrix can be "wells" or "strips" of a microplate, plastic beads or nitrocellulose paper. Conjugates are most often antibodies (IgG, sometimes IgM and IgA also) coupled to enzymes (alkaline phosphatase or horseradish peroxidase), fluorochromes or other reagents that will subsequently bring about a reaction that can be visualised. In case of enzyme conjugates the signal generated is a colour reaction and in case of fluorochrome it is fluorescence. The substrates used are 4-nitrophenylphosphate for alkaline phosphatase and o-phynxylencdiamine dihydrochloride (OPD) and TMB for horseradish peroxidase, which produce colour on being acted upon by the respective enzymes and the colour can be either detected visually or measured on a ELISA Reader as OD values.

All kit controls as specified in the package insert of the kit must be included with each test run to validate the test. Internal controls also must be included with each run to ensure quality results. Preparation of internal controls for practising quality assurance is detailed in the chapter 10 and 11.

6.9.2.1 Indirect ELISA

HIV antigens are attached covalently to the solid phase support allowing HIV antibodies present in the specimen to bind, and these bound antibodies are subsequently detected by enzyme labelled anti-human immunoglobulin and specific substrate system. If the test specimen contained antibodies colour reaction will take place.

Indirect ELISA is the most commonly used system.

General procedure

Fig. 6.2 gives the diagrammatic representation of indirect ELISA.

The instructions given in the package insert provided in the kit are read carefully and followed to the letter. All controls as specified must be included with each test run to validate the test result.

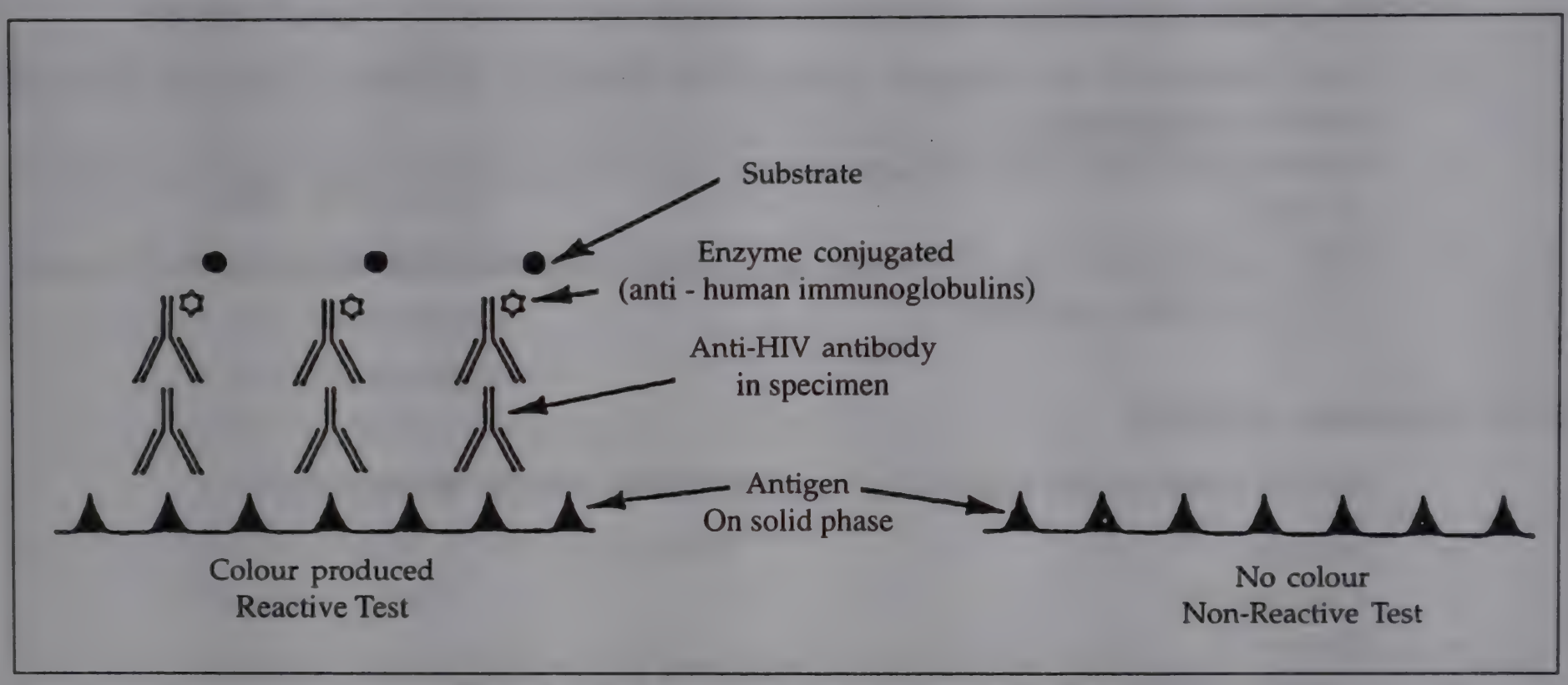


Fig. 6.2 Principle of the indirect ELISA

- Dilute specimen appropriately, add to the solid phase and incubate for a specified time and at specified temperature.
- Solid phase is washed to remove unbound antibodies
- Appropriately diluted enzyme conjugate is added and incubated as specified
- Solid phase is washed to remove excess conjugate
- Substrate made appropriately is added
- Colour change produced is measured after specified time using an ELISA Reader at wavelength specified
- The result is calculated as detailed in the package insert from the various OD values obtained.

The indirect ELISA produces a colour change directly proportional to the concentration of specific antibodies in the specimen.

6.9.2.2 Competitive ELISA

In this assay the HIV-antibodies present in the specimen compete with the enzyme conjugated antibodies in the reagent for binding to the antigen on the solid phase. If the test specimen contains HIV antibodies, these will compete with the labelled antibodies in the reagent for binding to antigen. So that less or no labelled antibodies can attach to the solid phase. Hence, faint or no colour is produced on addition of substrate if specimen contained HIV antibodies.

Unlike indirect ELISA, here reduction and or absence of colour indicates the presence of HIV antibodies in the test specimen. Development of strong colour means specimen is non reactive for HIV antibodies (**Fig. 6.3**).

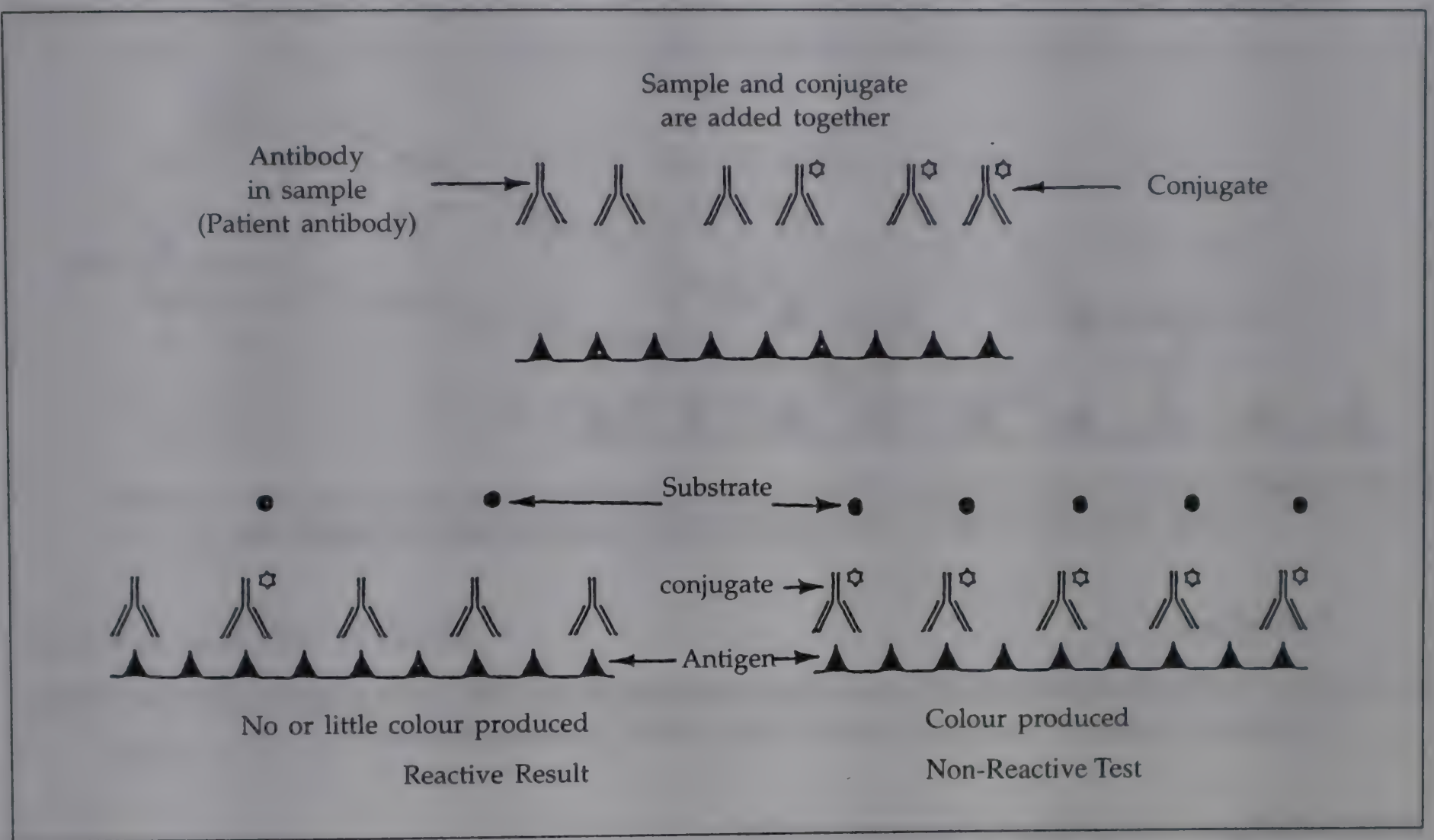


Fig. 6.3 Principle of the competitive ELISA

Procedure:

Follow the instructions given in the package insert.

- A dilution of test specimen and the appropriate amount of enzyme labelled HIV antibody conjugate are mixed and added simultaneously to the solid phase antigen support. Incubated for specified time at specified temperature.
- Plate is washed and substrate is added.
- Colour change developed is measured by ELISA reader and expressed as OD value.

Competitive ELISA takes less time than indirect ELISA and no predilution of test specimen is required.

6.9.2.3 Antigen and antibody sandwich ELISA

This is a modification of indirect ELISA to improve sensitivity and specificity of the test. Antigen bound to the solid phase binds antibody in the test specimen in first step. Since antibody molecules are bivalent they are still able to bind to another molecule. The next step is addition of similar enzyme labelled HIV antigen i.e. same antigen as on solid phase. This will attach to the antibody molecule which is already bound to the solid phase antigen with one arm (**Fig. 6.4**). Thus forms a sandwich of antigen + antibody + enzyme labelled antigen complex. The next step is addition of specific substrate which results in development of colour which is measured by ELISA reader. One big advantage of sandwich ELISA is that all classes of HIV-antibodies can be detected.

There is also antibody sandwich ELISA which is done to detect p24 antigen (described in Chapter 12).

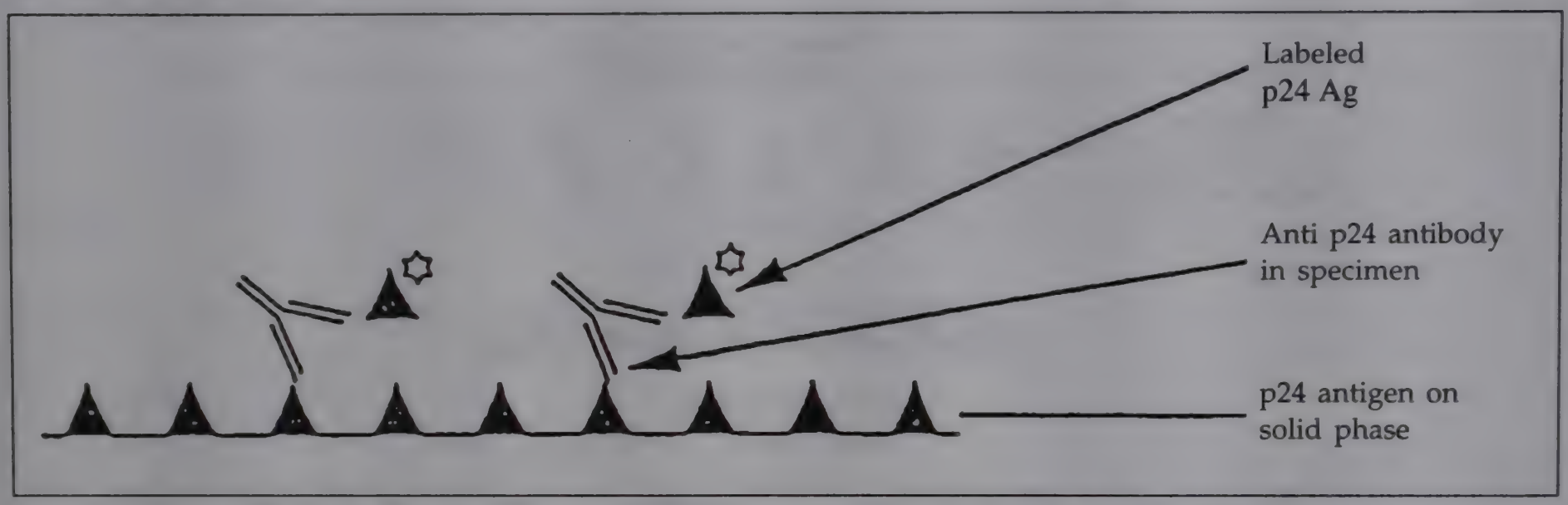


Fig. 6.4 An antigen sandwich ELISA for detecting HIV antibody

Procedure:

Same as indirect ELISA only difference being that in this case enzyme labelled antigen is added in place of enzyme labelled anti-human immunoglobulins.

6.9.2.4 Antigen and antibody capture ELISA

Antigen capture ELISA can be based on principle of indirect or competitive ELISA, only difference being in the initial step of attaching antigen to the solid phase.

A monoclonal antibody directed against an HIV antigen is bound to the solid support. Next step is addition of HIV antigen supplied as reagent. This antigen is captured by the monoclonal antibody bound to the solid phase. Test specimen appropriately diluted is added next. HIV antibodies if present in the specimen bind to HIV antigen on solid support. Remaining principle is same as indirect ELISA (**Fig. 6.5 and 6.6**).

Only advantage of antigen capture ELISA is that it is more specific than indirect assay.

Antibody capture assays were developed to test specimens with low concentration of HIV antibodies (e.g. urine and saliva) or to detect specific class of antibodies (e.g. IgG, IgM or IgA). In this test an anti-human immunoglobulin (anti-IgG, IgM or IgA) is attached to solid support. The patient specimen is added. The concentrated immunoglobulin in patient's specimen binds to the anti-globulins on solid phase. Next labelled antigen is added which

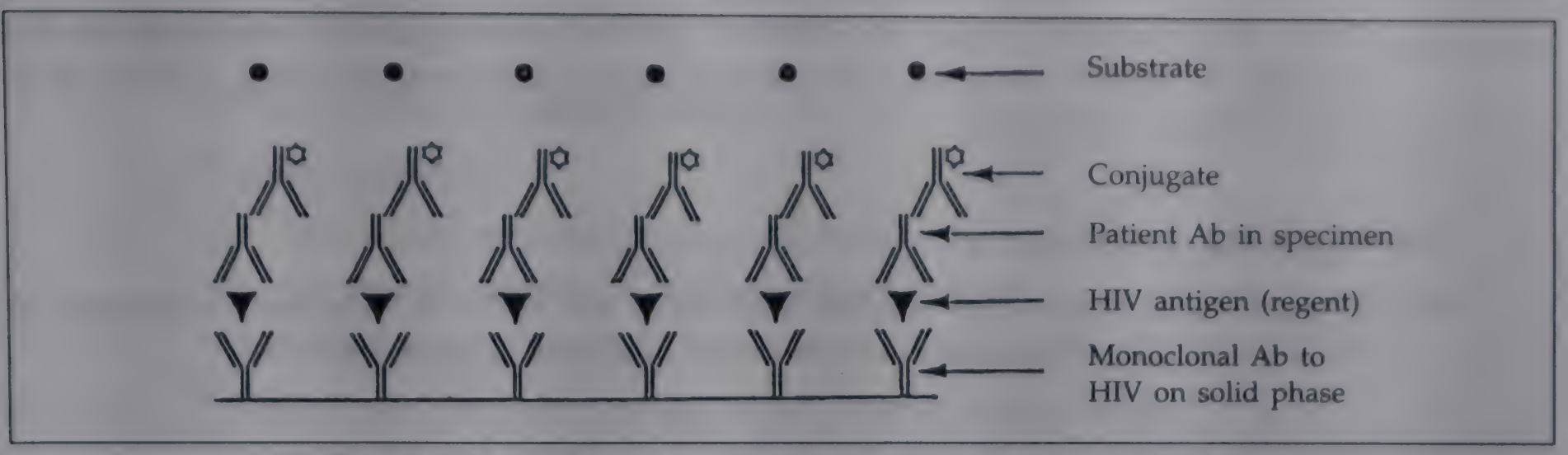


Fig. 6.5 Principle of the indirect antigen capture ELISA

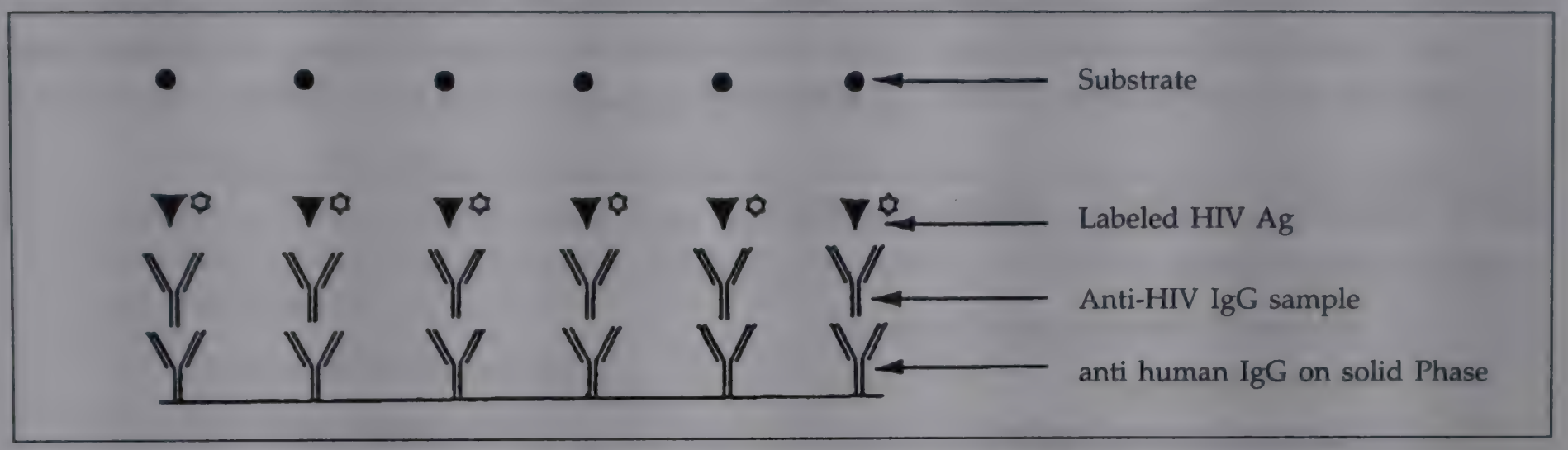


Fig. 6.6 The IgG antibody capture ELISA

binds to HIV antibodies of the patient bound to solid support. Next, the substrate is added and the OD value is read on the ELISA Reader.

6.9.3 Interpretation of results

Each test run must be validated according to the validation criteria given in the package insert of the kit.

Cut-off-value

- Each kit manufacturer has devised a method of calculation that produces a cut off value, to classify a test sample as positive or negative.
- This cut-off value can be based on an average of the negative controls multiplied by a factor or is based on a relationship of positive controls, to optimize sensitivity and specificity of assay.

Grey zone identification

- All infected individuals start with low levels of antibody and gradually the level of HIV antibody increases as the infection progresses. Therefore, some laboratories testing high risk, high incidence populations institute "grey zone" identification where specimens which are just (10%) below the cutoff (i.e., cut-off multiplied by 0.9) are classified as

"grey zone reactive", which are retested and subjected to confirmatory testing, like repeatedly reactive samples. This method may identify seroconverting patients early in the course of disease.

Repeat testing

- Reactive and "grey zone" reactive specimens are normally repeated in duplicate in ELISA and consensus results (2 or 3 test results) are accepted.

6.9.4 Performance variables/troubleshooting

ELISA tests are generally easy to perform but require careful adherence to procedures; any deviation in incubation times and/or temperature and pipetted volume can dramatically change test results (see Chapter 10 also).

6.9.4.1 Specimen problems – things to avoid

- Haemolysis
- Repeated freezing and thawing
- Gross lipaemia
- Bacterial contamination
- Heat inactivation – may cause falsely reactive ELISA

6.9.4.2 Operator/technical error

- Improper timing of incubations can give
  - low or high absorbance readings
- Incubation temperature
  - Low temperature – low absorbance readings
  - High temperature – high absorbance readings
- If conjugate precipitates → low reading: if heat inactivates enzymes → low reading

6.9.4.3 Equipment problems

- Incubator
  Poor air circulation (hot spots, cold spots)
  Stacking plates (>2); improper heating
- Water Bath
  Contaminated water, splashes into plates or tubes
- Improperly maintained plate washer
  Improper washing, high O.D. background, left over conjugate on plates creates colour change.
- Improperly calibrated/maintained microplate reader

6.9.4.4 Operator technique

Variation within or between plates

- Improper washing
  (a) Incomplete removal of wash solutions – low absorbance readings.
  (b) Inadequate washing – high absorbance readings non-specific reactions.
- Inaccurate/incorrect pipetting – absorbance readings, high or low.
- Failure to add sample controls, or reagents to correct wells.
- Timing of reagent additions, especially stop solution in relation to substrate.
- Reagents reconstituted or diluted in wrong diluent or wrong volumes.

6.9.5 False positive and false negative ELISA results

There are some conditions other than HIV infection which may give a reactive HIV results i.e. false positive result. Also, sometimes the result may be negative even in HIV infected i.e. false negative result. Some of the common conditions giving erroneous results are listed below.

- Auto-immune diseases
- Multiple pregnancies
- Multiple transfusions
- Antibody to Class II HLA Ag (HLA-DR4)
- Hyper agammaglobulinaemia
- Antipolystyrene antibodies
- Chronic alcoholics
- Patients with hepatitis
- Hepatitis B immunisation
- Technical error etc.
- Others

False negative

- Infected but not yet seroconverted, window period
- Late stage disease (immune collapse)
- Technical error

6.10 RAPID ASSAYS

A number of rapid assays based on principle of agglutination and ELISA have been developed for ease of performance and quick results. These assays generally require less than 30 minutes to perform and do not require special equipments.

6.10.1 Agglutination assays

Agglutination assays incorporate a variety of antigen coated carriers like red cells, latex particles, gelatin particles and microbeads. These particles are used to support or carry the antigen. HIV antigens are attached to the carrier particles by non specific attachment.

Agglutination assays have good sensitivity, do not require sophisticated equipment, are easy to perform, require no wash procedures and are cost effective. However, specificity is somewhat compromised and prozone reaction may be seen. To overcome the prozone reaction, diluted specimen is used to perform the test. During the agglutination reaction HIV antibody combines with HIV antigen on the carrier particles and since all antibodies are multivalent, a sort of lattice network is formed which can be visualised macroscopically or microscopically as per the directions of the manufacturer.

Procedure

The instructions given in the package insert should be strictly followed.

- A dilution of test specimen is mixed with HIV antigen containing carrier particles.
- If test specimen contains HIV antibodies, a lattice network will form between the antigen carrying particles and antibodies and is visualized as formation of clumps (**Fig. 6.7**).
- Agglutination is seen visually except when carrier particles are microbeads.
- Weak reactions read subjectively must be confirmed by ELISA.

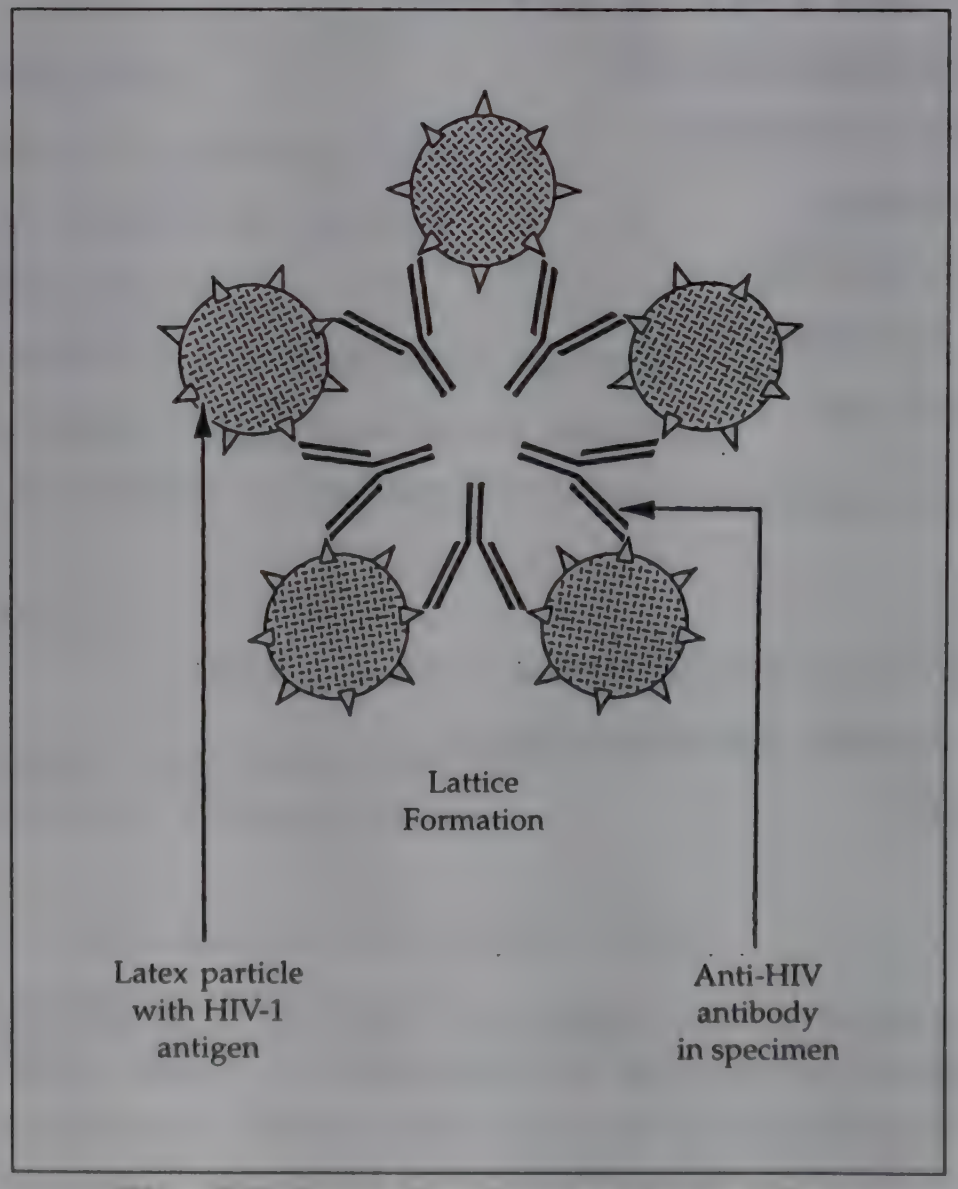


Fig. 6.7 Agglutination assay positive

6.10.2 Dot blot assays/Comb tests

These assays are rapid, easy to perform, can usually discriminate between HIV-1 and HIV-2 and do not require sophisticated equipment. The results are read by development of colour. Only drawback of the test is the high cost. Sensitivity and specificity of these assays compares with ELISA.

The assays utilize recombinant or synthetic peptides spotted on to nitrocellulose paper/ micro particles. The antigen containing matrix is housed in a plastic device (cassette) containing adsorbent pads underneath to collect reactants or made as a comb and the antigens are spotted onto the tooth of the comb card (Chapter 7). Each assay contains an immunoglobulin capture control to validate the result. These assays are very good for single test application i.e. in emergency, autopsy room and peripheral blood banks. They have not been automated so far.

Procedure (comb and dot blot tests)

The instruction given in the package insert should be followed.

- Dilution of test specimen is added which filters through the membrane/microbeads by action of gravity. During the process HIV antibodies in the test specimen bind to HIV antigens on the membrane.
- Wash solution is added to remove unbound antibodies.
- The appropriately diluted conjugate is added (antihuman – immunoglobulin conjugated with enzyme as in indirect ELISA or protein A conjugated with colloidal gold particles. Protein A conjugate can bind to human IgGs directly).
- Rinse with wash solution to remove excess of reactants.
- Substrate appropriately diluted is added which forms a precipitate at sites on membrane which contain antigen antibody complexes.
- Colour development in the control and test dots is read visually.

Fig. 6.8 & 6.9 show diagrammatic representation of comb and dot blot assays

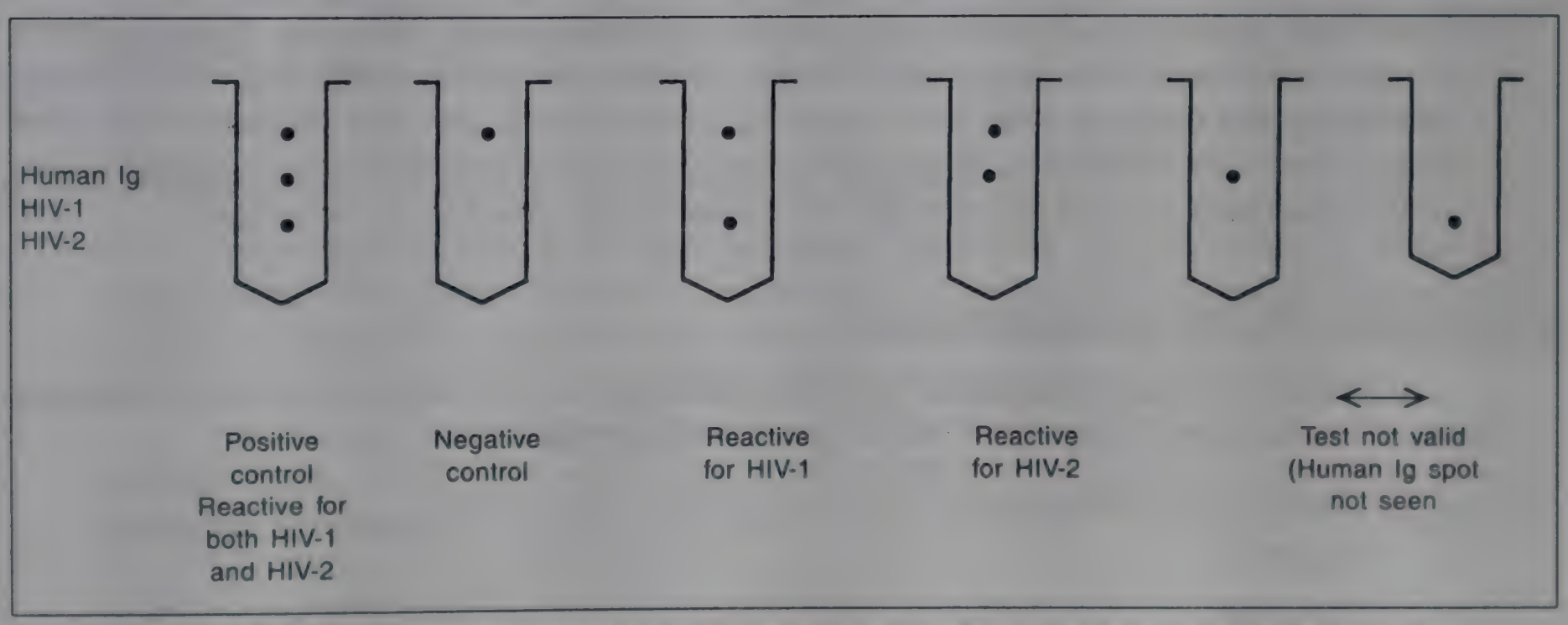


Fig. 6.8 Diagrammatic representation comb test (Tooth of comb card shown)

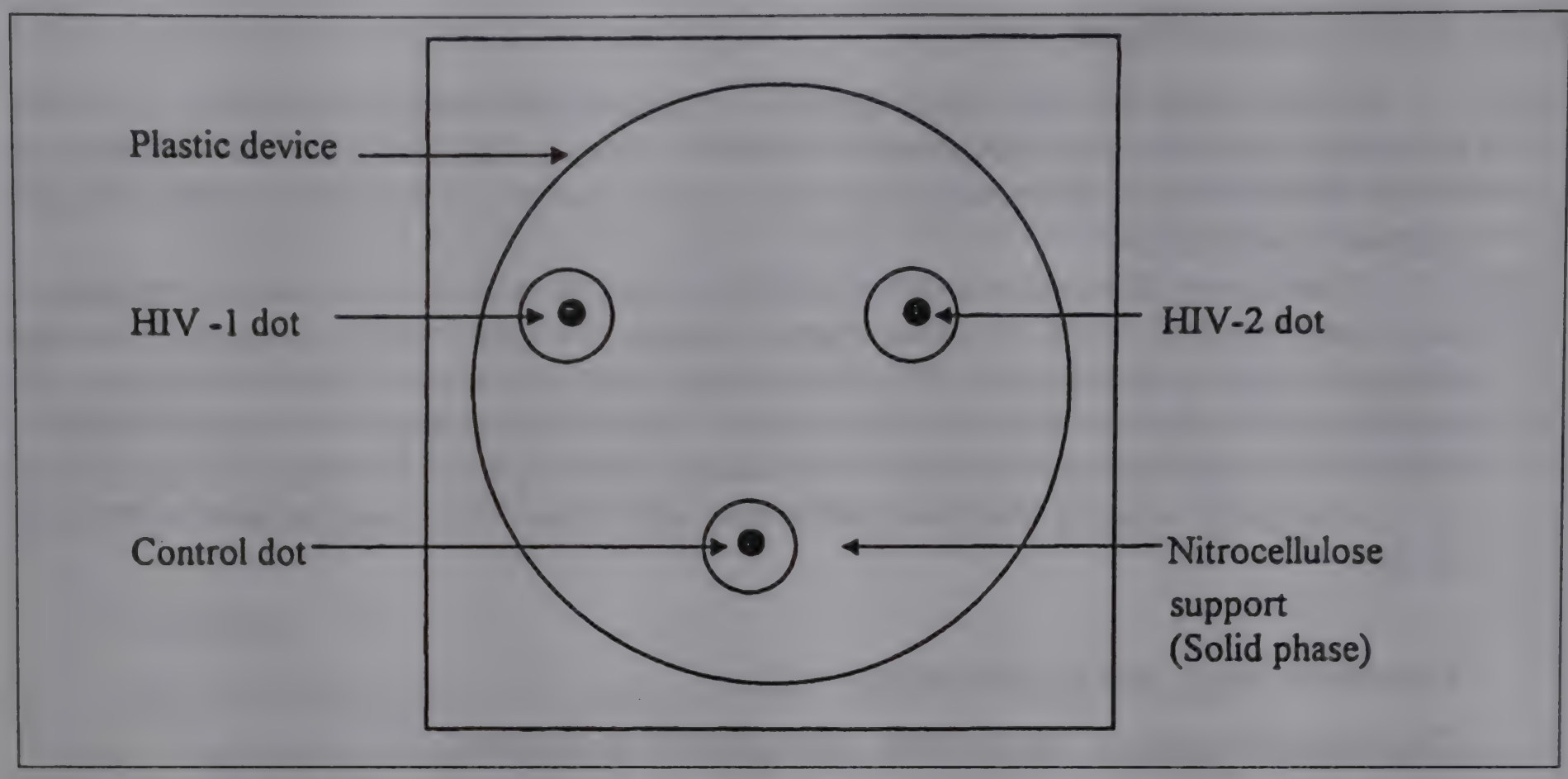


Fig. 6.9 A combination HIV-1/HIV-2 dot-blot assay to differentiate infection

Interpretation of result is done as per the instructions in the package insert. However, reactive results have to be confirmed by ELISA.

Immunocomb test is similar to blot assay based on principle of indirect ELISA. Here also the instructions given in the package insert with the kit are followed to perform the test.

6.11 HIV ANTIBODY DETECTION IN OTHER FLUIDS

The standard specimens for detection of HIV antibodies are serum/plasma/blood. However, detecting HIV antibodies in other fluids is also being developed. HIV antibodies can be detected in oral fluids (saliva/oral mucosal transudates). The levels are less than 1% of the levels in serum, but can be detected by sensitive ELISA technology. The isotype of antibody detected is secretory IgA, so appropriate ELISA should be used. The tests have shown high sensitivity and specificity as per the reports from U.S., Tanzania, Thailand, Brazil and other countries. These tests may find better use once the issues concerning confidentiality, counselling and follow up have been resolved. HIV antibodies can also be detected in urine using appropriate ELISA kits. These tests have also been validated. However, guidelines for using these tests as well as strategies for follow up etc. need to be developed.

6.12 CHOICE OF THE SCREENING ASSAY

Before choosing a particular protocol/kit/strategy for HIV testing one has to be clear about the following parameters before testing is undertaken.

- Objectives of testing
- Sensitivity
- Specificity

- Prevalence of HIV infection in the population.
- Cost effectiveness.
- Appropriateness to the strategy and national guidelines of testing and according to the infrastructure facilities available (Chapter 9).

6.12.1 Objectives of HIV testing

- Blood and blood products safety. This is achieved by mandatory testing of all donated blood units and blood products.
- Screening of donors of sperms, organs and tissues.
- Diagnosis of HIV infection in clinically suspected cases.
- Voluntary testing after counselling.
- Epidemiological surveillance using unlinked anonymous HIV testing. Here the result of test cannot be linked with the identity of the person.
- Research.

6.12.2 Sensitivity of HIV test

It is the accuracy with which a test can confirm the presence of an infection. Tests with high sensitivity show few false negatives and are meant to be used to screen blood prior to transfusion and ensure blood safety.

$$\text{Sensitivity} = \frac{\text{True positive (TP)}}{\text{TP + False negative (FN)}} \times 100$$

6.12.3 Specificity of HIV test

It is the accuracy with which a test can confirm the absence of an infection. Tests with high specificity show few false positives and are to be preferred for the diagnosis of HIV infection in an individual.

$$\text{Specificity} = \frac{\text{TN (True negative)}}{\text{TN + FP (False positive)}} \times 100$$

6.12.4 Prevalence of HIV and its implication for test results

The probability that a test will accurately determine the true infection status of a person being tested varies with the prevalence of HIV infection in the population from which the person comes. The higher the prevalence, greater is the probability that a person testing positive is truly infected i.e. greater the positive predictive value of the test (PPV).

The likelihood that a person showing a negative result is truly uninfected the negative predictive value (NPV) decreases as the prevalence of HIV infection among the general population increases. The details for calculation of PPV and NPV are given in Chapter 11.

UNAIDS and WHO recommendations for HIV testing strategies according to test objective and prevalence of infection in the sample population		
Objective of testing	Prevalences of infection	Testing strategy
Transfusion/transplant safety	All prevalences	I
Surveillance	>10% ≤10%	I II
Diagnosis: Clinical signs/symptoms of HIV infection	>30% ≤30%	I II
Asymptomatic	>10% ≤10%	II III

6.12.5 Strategies of HIV testing in India

Because of the enormous risk involved in transmission of HIV through blood, safety of blood and blood products is of paramount importance. Since the PPV is low in populations with low HIV prevalence, WHO/GOI have evolved strategies to detect HIV infection in different population groups and to fulfil different objectives (Annexure 6.1). The various strategies, so designated, involve the use of categories of tests in various permutations and combinations.

1. ELISA/Simple/Rapid tests (E/R/S) used in strategy I, II & III
2. Supplemental test like Western Blot and Line Immunoassay are used in problem cases e.g. in cases of indeterminate/discordant result of E/R/S.

Strategy I: Serum is subjected once to E/R/S for HIV. If negative, the serum is to be considered free of HIV and if positive, the sample is taken as HIV infected for all practical purposes. This strategy is used for ensuring donation safety (blood/blood products organ, tissues, sperms etc.). The unit of blood testing reactive (positive) is discarded. Donor is not informed.

Strategy II: A serum sample is considered negative for HIV if the first ELISA report is so, but if reactive, it is subjected to a second ELISA which utilizes a system different from the first one. It is reported reactive only if the second ELISA confirms the report of the first. This strategy is used for surveillance and for diagnosis only if some AIDS indicator disease is present.

Strategy III: It is similar to strategy II, with the added confirmation of a third reactive ELISA test being required for a sample to be reported HIV positive. The test to be utilized for the first ELISA is one with the highest sensitivity and for the second and third ELISAs, tests with the highest specificity are to be used.

Strategy II & III are to be used for diagnosis of HIV infection. ELISA 2 and ELISA 3 ought to be tests with the highest PPV possible to eliminate any chances of false positive results. Strategy III is used to diagnose HIV infection in asymptomatic individuals indulging in high risk behaviour.

6.13 SUPPLEMENTAL (CONFIRMATORY) ASSAYS

Supplemental/confirmatory test are undertaken to confirm the HIV infection status of an individual who is asymptomatic and has history of high risk behaviour.

Supplemental assays are used for purpose of diagnosis of HIV infection and identification of the individual i.e. the individual is informed about the test results in the proper way (counselling and confidentiality). This is because the screening assays are highly sensitive and are bound to give biologic false positive reactions whereas supplemental assays are sensitive as well as specific and are performed to rule out false positive reactions. Supplemental E/R/S using different antigen and/or principle of test are performed to fulfil the strategy II and III of HIV testing policy.

However, supplemental tests do not always give conclusive results in which case either other tests (PCR, p24 antigen detection etc.) have to be resorted to or follow up study is practised.

Various supplemental assays available commercially are listed below:

- ELISA with different antigen system (recombinant or synthetic peptides) or with different principle of test which makes the test more specific;
- Western blot (WB);
- Immunoblot (IB);
- Line immunoassay;
- Indirect fluorescent antibody test (IFA);
- Radioimmunoprecipitation test (RIPA).

As per the National HIV testing policy of India, supplemental tests are used only for strategy II and strategy III of HIV testing. For the purpose, E/R/S with higher specificity can be used as supplemental tests, provided these assays incorporate different antigen system and/or different principle of test. Only in case of unequivocal/discordant result WB is to be used. This is to make the HIV testing cost effective as well as easy to perform. The other supplemental tests i.e. IFA, RIPA are hardly ever used in India. So, only the supplemental assays used in India are being described.

6.13.1 E/R/S tests as supplemental assay

Supplemental E/R/S are recommended for specimen reactive in the screening assay for strategy II and III of HIV testing policy. If the screening assay contained virus lysate as antigen the second and third assays should have recombinant or synthetic peptides as antigens. Use of recombinant and synthetic peptides makes the assay more specific while retaining the sensitivity. Alternatively the second and third assays can also be based on different principle of ELISA e.g. if the screening assay was indirect ELISA the supplemental assays should be based on principle of competitive ELISA/Sandwich ELISA etc., which again is done to increase the specificity of the screening assay when used as supplemental assays.

6.13.2 Western blot/Immunoblot/Line immunoassay

These are most widely accepted supplemental assays, are highly specific but are expensive, labour intensive, need expertise to interpret and may give unequivocal/indeterminate results. The specificity of these tests is based on two factors: separation of antigens and their concentration.

IB and WB use viral antigens from whole virus lysates electrophoretically transferred to a membrane support. So these blots may contain contaminating cellular components.

Recombinant or synthetic HIV antigens mechanically applied onto the support membrane are used in Line Immunoassays (LIA). These do not contain contaminating cellular components and are highly specific.

Indeterminate results can be seen by any of the above assays.

WB/LIA HIV kits are commercially available.

6.13.2.1 Procedure

The instructions given in the package insert must be diligently followed both for performance of test as well as interpretation of result.

General Procedure:

- HIV Ag test strips are placed in individual troughs of plastic tray.
- A dilution of the patient serum/plasma or control is added to individual test troughs and incubated on a rocking platform.
- At the end of incubation, the troughs are aspirated and the test strips are washed to remove unbound antibodies.
- An enzyme conjugate antihuman immunoglobulin is added to bind to human antibodies bound to HIV antigens on the test strips.
- The troughs are aspirated and strips are washed to remove unbound conjugated immunoglobulins.
- A substrate is added and colour development occurs where antigen/antibody complexes are localized on the strips.
- The colour development is stopped; the strips are washed, dried and interpreted.

Fig. 6.10 gives diagrammatic representation of WB test.

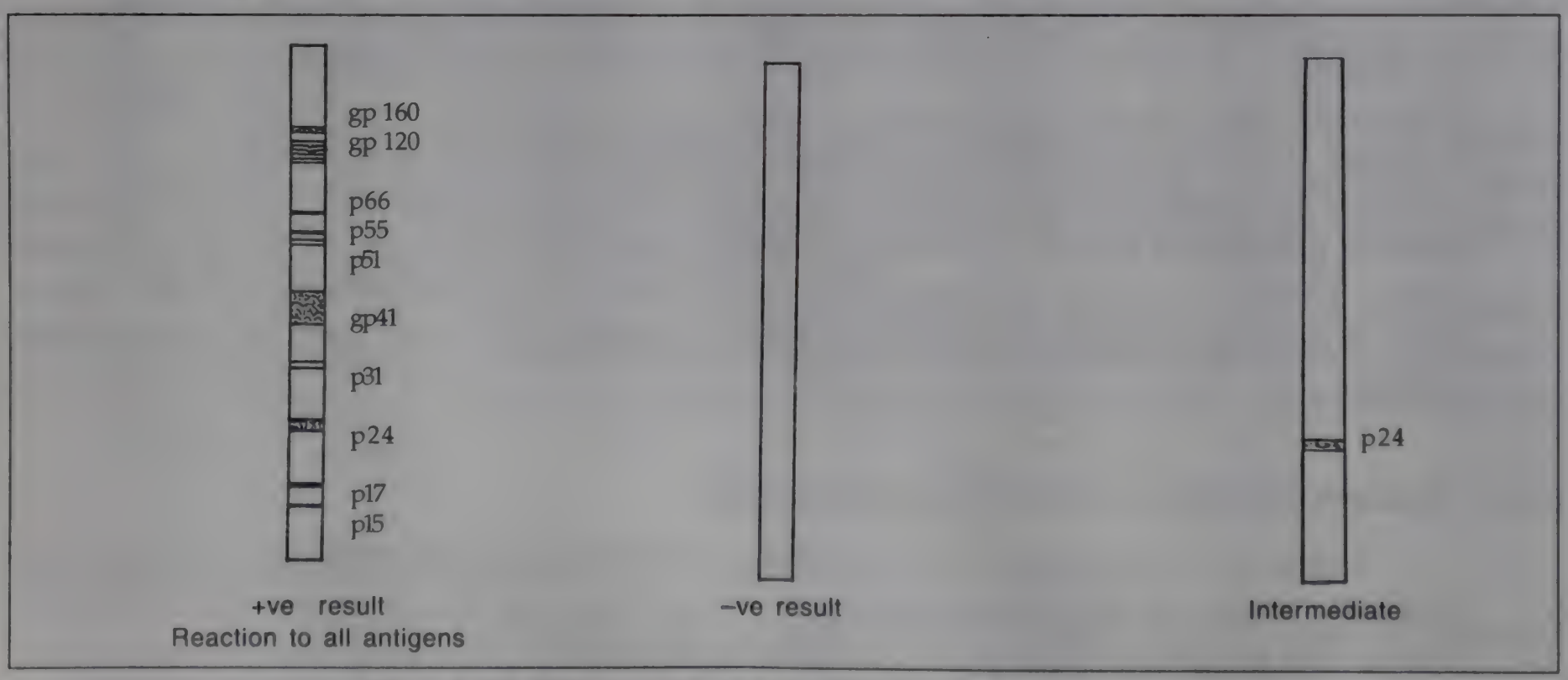


Fig. 6.10 Schematic representation of WB results

6.13.2.2 Reading and Interpretation

The presence or absence of bands of the major viral proteins is scored for each strip. Unusual or atypical band locations are also noted.

Interpretative Criteria

Results are interpreted either as per WHO criteria i.e. presence of at least two envelope bands (gp 120, gp160, gp41 etc.) or as per the manufacturer's criteria for positive, indeterminate and negative.

In case of indeterminate result, retesting of the individual is done after two weeks, 1 month, 3 months and sometimes upto 1 year. During this time either the test becomes negative or positive or may stay indeterminate. Decision about the result has to be reached after correlation with history of high risk behaviour and clinical parameters.

6.14 LABORATORY DIAGNOSIS OF HIV INFECTION IN NEWBORN (CONGENITAL HIV INFECTION)

Transplacental transmission of HIV can occur from infected pregnant mother to the foetus as early as 8 weeks of gestation or may be even earlier. It is estimated that > 80% of AIDS cases in infants are due to perinatal transmission of HIV-1. Diagnosis of HIV infection in infants born to seropositive mothers is difficult because maternal antibody (IgG) to HIV-1 crosses the placenta and can persist for upto 15 months making the distinction between maternal and neonatal IgG difficult. The tests which can be undertaken to diagnose HIV infection in neonates before 15 months of age are detailed below.

6.14.1 Detection of IgA and/or IgM anti-HIV antibodies

These antibodies do not cross placenta. The IgA class of HIV antibody assay using Western blot technique in infected children at 3 months of age has a sensitivity of 97.6% and specificity of 99.7% as reported in a study. IgM class of antibodies are produced by infected infants by six months of age. Production of IgM is erratic, false positive results are obtained due to rheumatoid factor and polyvalent nature of IgM leading to nonspecific binding.

6.14.2 Estimation of p24 antigen (core antigen)

The immune-complex dissociation assays which involve pretreatment of serum/plasma to liberate p24 antigen complexed with p24 antibody prior to performance of ELISA are quite sensitive in identifying HIV-infected infants.

6.14.3 Polymerase chain reaction (PCR)

The technique specifically amplifies viral DNA sequences of interest. Theoretically it is possible to identify one infected cell in the specimen as also latent HIV infections. Various reports indicate the specificity of PCR to be invariably >95% regardless of age of testing while sensitivity ranges from 15% in neonates (within 48 hrs. after birth) to more than 95% in infants over 1 month of age.

6.14.4 In-vitro antibody production assay

The method is tedious and involves in-vitro culture of antibody producing B lymphocytes from peripheral blood. The HIV antibodies are detected in culture supernate from truly infected infants.

6.14.5 In-vitro isolation of virus from blood or tissues

The method is time consuming, expensive and not sensitive. This is due to the lower number of available cells cultured and paucity of infected cells in the sample that are present. Additionally, it may take upto 6 weeks or longer to obtain a result. Very few laboratories in India are equipped to culture HIV.

6.14.6 Indirect indicators of HIV infection

These include various nonspecific markers like hypergammaglobulinemia, absolute lymphopenia, hematologic abnormalities, low CD4 cell counts and clinical markers like severe failure to thrive or wasting syndrome, recurrent severe bacterial infections, opportunistic infections, HIV associated malignant disease, encephalopathy, lymphocytic interstitial pneumonia, etc. which point towards acquired immunodeficiency syndrome. However, these parameters are employed to supplement the conventional serodiagnosis of HIV infection. The tests available for diagnosis of vertical transmission of HIV from infected mother to the neonate err on the side of high specificity with a lower sensitivity.

6.15 LABORATORY DIAGNOSIS DURING WINDOW PERIOD

HIV infection during window period can be detected by demonstrating the presence of virus and virus components.

- PCR (procedure details given in
- p24 antigen assay (<40%) Chapter 8 and Chapter 12)
- Viral Culture

Need of laboratory diagnosis in window phase

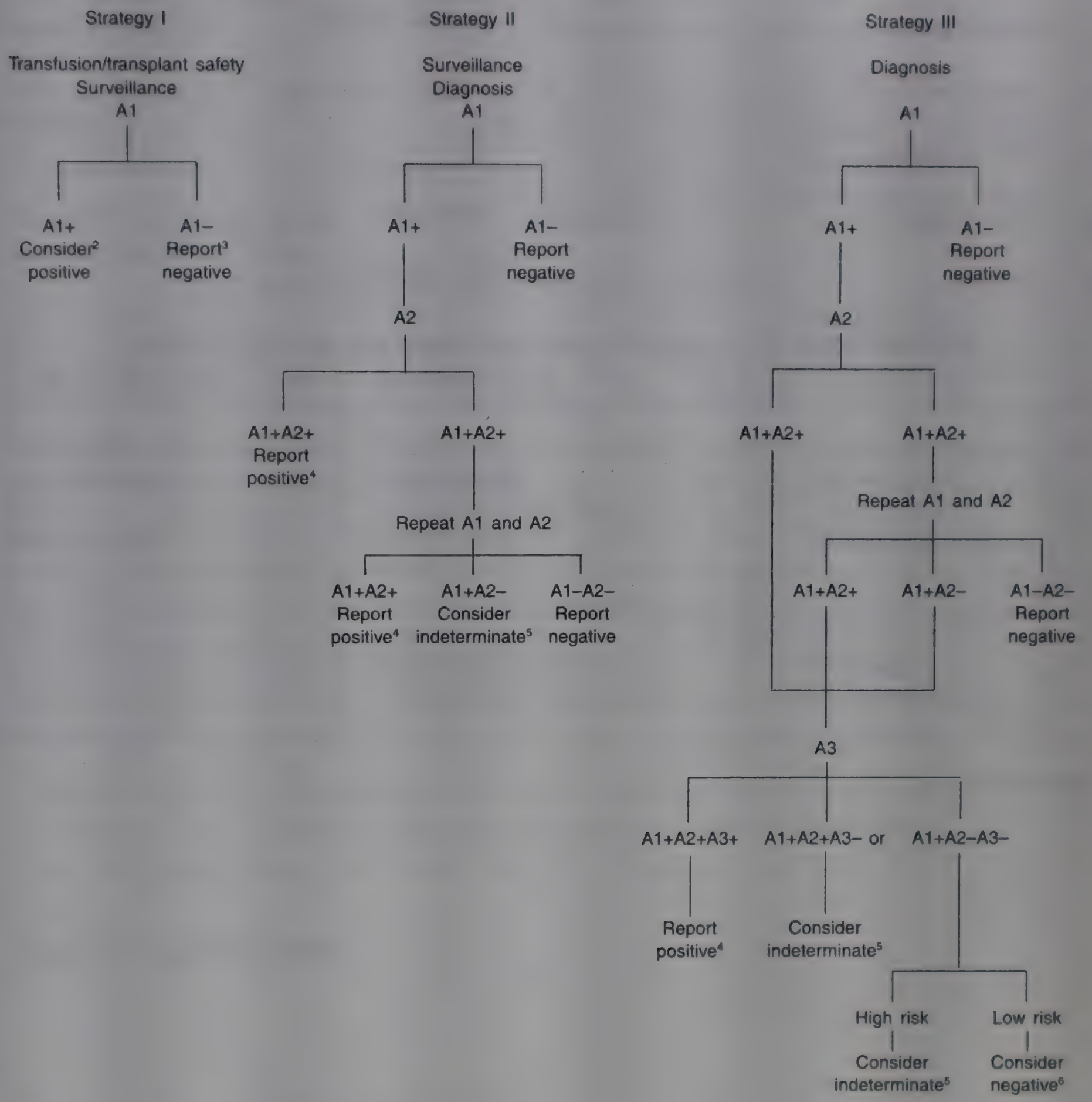
- Following untested blood transfusion.
- Risky heterosexual/homosexual exposure.
- Needle stick injury (contaminated)

Annexure 6.2 A model laboratory proforma for performing ELISA

Annexure 6.3 Proforma (information) for requisitioning HIV testing.

Chapter 6 - Annexure 1

Schematic representation of the UNAIDS and WHO HIV testing strategies



[1]Assay A1, A2, A3 represent 3 different Assays
[2]Such a result is not adequate for diagnostic purposes: use strategies II or III. Whatever the final diagnosis, donations which were initially reactive should not be used for transfusions or transplants.
[3]Report: Result may be reported.
[4]For newly diagnosed individuals, a positve result should be confirmed on a second sample.
[5]Testing should be repeated on a second sample taken after 14 days.
[6]Result is considered negative in the absence of any risk of HIV infection.

Chapter 6 - Annexure 2

A model laboratory proforma for performing ELISA

1. Referred by

2. Sample number

3. Name of the kit:

4. Lot #:

5. Date of expiry:

6. Date of test run:

7. Technician:

8. Results:

CUT-OFF VALUE =

INSTRUCTIONS: Fill in sample number and results into appropriate space												
	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Comments: _____

_____

_____

Officer Incharge (Laboratory)

Chapter 6 - Annexure 3

Proforma (information) for requisitioning HIV testing

Request for HIV Test
Lab. No. .....
Dated ..... Blood Taken on:
Received ..... Date

REQUEST FOR HIV ANTIBODY
Previously Tested: Yes ☐ When ..... Neg ☐
No ☐ Where ..... Pos ☐

Surname (Capitals) Forenames Hospital Regd. No.
.....

Age M/F

Occupation
. .....
Ward/Home Add./Hosp.
.....

Request for HIV made by Consultant/G.P. (with address for report)
.....
.....
.....
Telephone No. .....

CLINICAL DETAILS

No Symptoms .....
Lymphadenopathy .....
AIDS .....
Others (specify).....
.....
.....
.....
.....

EPIDEMIOLOGY

Homo/bisexual	☐	☐
Haemophiliac	☐	☐
Intravenous drug absuer	☐	☐
Blood recipient	☐	☐
Prostitute	☐	☐
Known AIDS or B.P. HIV Pos.	☐	☐
Heterosexual only	☐	☐

Suspected Country of Infection .....

CONTACT OF

Healthy Visiting Abroad ☐
Semen Donor ☐
Organ Donor ☐
Others .....
.....
.....
Nationality .....
Passport No. .....

Chapter 7

DIFFERENTIAL FEATURES IN EPIDEMIOLOGY, NATURAL HISTORY, VIROLOGY, PATHOGENESIS AND LABORATORY TESTING OF HIV-2 INFECTION

7.1 EPIDEMIOLOGY

HIV-2 was first discovered in Senegal, West Africa in 1985. During the past decade significant HIV-2 infection has been reported from West Africa, Portugal, Mozambique, Angola, Cuba, Brazil. Important countries of West Africa namely Guinea-Bissau, Burkinafasso, Ghana, Ivory Coast, Nigeria, Mali reported high prevalence of HIV-2 infection. HIV-2 has also been reported from Europe, U.S.A. and Canada. The first documentation of HIV-2 in India was made from Bombay in 1990. There are reports of HIV-2 infection from Maharashtra, Delhi, Chennai, Visakhapattanam, Uttar Pradesh, Bihar, Punjab and Manipur.

7.2 VIROLOGY

HIV-2 is similar to HIV-1 biologically and has similar host range, morphology and genetic organisation. Comparison of major gene products of HIV-1 and HIV-2 is given below:

Gene	HIV-1	HIV-2
gag		
Precursor	p55	p56
Core	p24	p26
Matrix	p17	p16
	p9	
	p7	
env		
Precursor	gp160	gp140
External	gp120	gp105/125
Transmembrane	gp41	gp36/41
pol		
Reverse Transcriptase	p66	p68
Reverse Transcriptase	p51	p53
Endonuclease	p31	p34

- Antigenically, most cross reactions in HIV serological assay are due to HIV antibodies directed against gag and pol antigens, resulting from the extensive nucleotide homology of these structural genes between HIV-1 and HIV-2.
- env cross reactions are less common.
- One of the regulatory components vpx (p16) is present in HIV-2 and not in HIV-1 and hence unique gene for HIV-2. It may be responsible for the different pathologic effects

noted with HIV-2 infections. This may have important diagnostic application since detection of anti vpx will identify HIV-2 infection especially when the sera react by both assays. Antibodies to the nef regulatory protein which may not function as down regulator in HIV-2 have been detected in 25% of HIV-2 infected individuals.

7.3 NATURAL HISTORY AND PATHOGENESIS

Mode of transmission of HIV-2 is same as HIV-1 but the rate of HIV-2 transmission is significantly lower. The most common routes of HIV-2 transmission are perinatal and heterosexual. Immunological and clinical observations have shown that there is slow progression of HIV-2 disease compared to HIV-1. The lower level of viral load may be related to the lower efficiencies of CD4 receptor binding and may be an explanation for the seemingly slower transmission and slower disease development in HIV-2 infection as compared to HIV-1. Some isolates of HIV-2 can infect CD4 cells by using fusin/CXCR4 molecules independent of CD4 receptor.

The differences in pathogenesis and other characteristics of HIV-2 as compared to HIV-1 infections are detailed below:

- Slow clinical deterioration and disease development;
- Fewer immunological disturbances;
- Low transmissibility;
- Longer incubation period;
- Lower rate of vertical transmission;
- Possible protection of HIV-1 infection;
- Low rate of efficiency of virus isolation;
- Low rate of opportunistic infections;
- Lower plasma tumour necrosis factor $-\alpha$;
- Lower viral load;
- Lower level of proviral DNA in circulating lymphocytes.

7.4 DUAL (HIV-1 AND HIV-2) ASSAYS

These can be either dual screening assays or dual confirmatory assays.

7.4.1 Dual screening assays

Dual screening tests are available in the form of ELISA and rapid tests. Most second generation dual screening tests use the indirect format and do not identify which antibodies are present (anti HIV-1 or HIV-2). A positive reaction only indicates that antibodies to one or the other are present. Third generation dual assays have been developed in both microtitre and bead formats. The dual screening tests are used for screening of blood donors or other individuals at unknown risk for HIV-1 and/or HIV-2 infection and for clinical diagnostic testing.

In ELISA, the principle is same as that of HIV-1 except that the antigens (recombinant or synthetic) of HIV-1 and HIV-2 both are coated in the same microwell. Rapid tests like dot

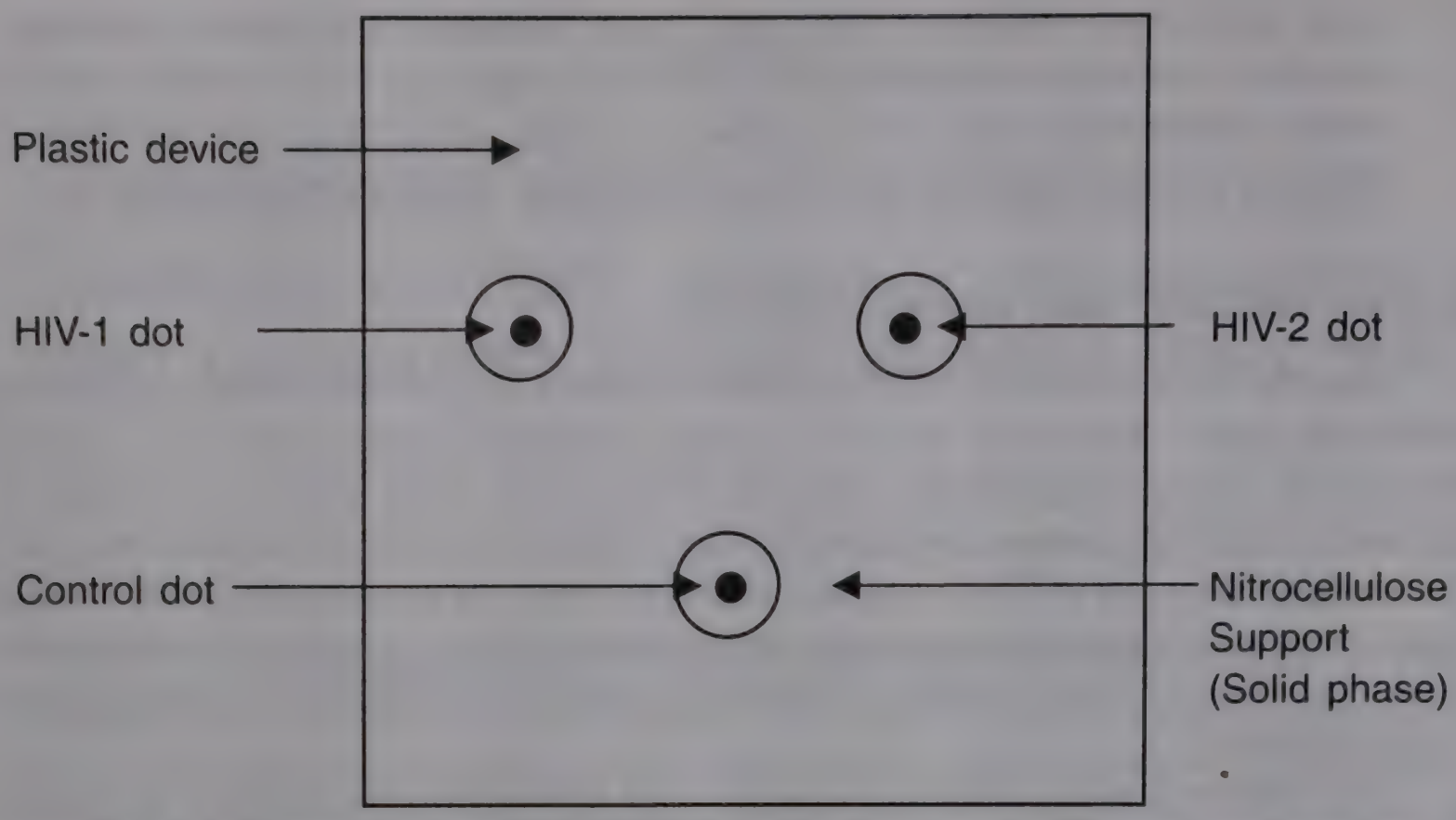


Fig 7.1 A combination HIV-1/HIV-2 dot-blot assay to differentiate infection

blot combination assays are available that have separate dots containing antigens for each virus. With these tests, differentiation of HIV-1 and HIV-2 infections can be accomplished with one test (**Fig. 7.1**).

The Immunocomb Bispot HIV-1 and 2 kit is based on the principle of the solid- phase ELISA. The plastic immunocomb card functions as the solid phase. Synthetic peptide antigens of HIV-1 and HIV-2 are spotted on to the projections of the card. There are rapid qualitative dual screening tests (HIV Spot) which involve trapping of antibodies to HIV-1 and/or HIV-2 by the capture reagents which are absorbed on a porous membrane. After washing, the presence of antibodies is revealed by treatment with a proprietory protein A-gold conjugate which will bind to absorbed HIV antibodies and form a red colour on the membrane. The capture antigens are envelope proteins of HIV-1 plus a highly purified peptide which corresponds to a region of the envelope transmembrane protein of HIV-2. In Capillus HIV-1/HIV-2 screening test, the envelope proteins of HIV-1 and HIV-2 are bound to polystyrene latex beads and form the basis of a direct latex aggregation assay for the detection of antibodies to HIV-1/HIV-2 in serum or plasma. The assay is performed on a patented capillary slide. For, diagnostic purposes, reactive results by these screening tests should be confirmed by supplemental/confirmatory tests.

7.4.2 Dual supplemental/confirmatory tests – Western Blot and Line Immunoassays, etc.

WB assays have been developed that have the ability to identify and differentiate infections by HIV-1 and HIV-2. Most incorporate the use of viral lysates from HIV-1 and synthetic peptides of HIV-2 artificially applied on the same nitrocellulose strip. Multiple HIV-1 antigens and one HIV-2 specific band (gp36 or gp41) are applied to the strip; antibodies to the HIV-2 antigen are considered to be diagnostic if a reaction is present. The blot also contains a control line, this indicates that the test has been performed properly (**Fig. 7.2**). Each company that markets these tests indicates in their package insert the criteria required for positivity. The criteria established by some manufacturers include reactions to one gene product from each of the three major genes (gag, pol and env) for positivity for HIV-1. To be

considered positive for HIV-2, reaction to the HIV-2 specific antigen plus a reaction to HIV-1 specific antigens (but which do not meet the criteria for positivity of HIV-1) must be present.

Dual tests have become available that contain artificially applied antigens from both HIV-1 and HIV-2 in a WB format. The Line Immuno Assays (LIAs) have gained the popularity and are based on the application of recombinant and synthetic peptide antigens on a plastic support strip in a manner similar to the Immunoblot Assay. The LIAs are second or third generation assays and have the potential for use as supplemental tests. Most LIAs utilise the indirect ELISA methodology for visualising the results. The major differences between the LIA and WB are that the antigens are applied, rather than electrophoresed, and are derived from recombinant or synthetic peptide technology rather than viral lysates. In the LIA, optimal quantities of antigens can be applied, resulting in an increased ease of reading and interpretation. The use of these antigens helps to eliminate the problems of indeterminate and atypical results that may occur due to antibodies to cellular components in viral lysate based confirmatory assays. These assays also allow for the application of antigens from more than one virus, thereby allowing them to act as combination assays and to differentiate infection by HIV-1 and HIV-2. The LIAs also contain control lines that indicate that the test is performed properly, and can act as a guide in estimating the intensity of reactions and helping in interpretation. One particular combination assay includes three recombinant HIV-1 proteins (p24, p17, p31) and two synthetic peptide antigens, one corresponding to the envelope antigen of HIV-1 (gp41) and one to the envelope antigen of HIV-2 (gp36). The LIA is easy to perform, requires a total time of 3 to 4 hours and performs well with blood collected on filter paper and on saliva samples. An example is given in (**Fig. 7.3**).

In case of discordant results, final confirmation will be done by follow up of the individual and molecular tests like site directed serology, nucleic acid hybridisation and PCR techniques.

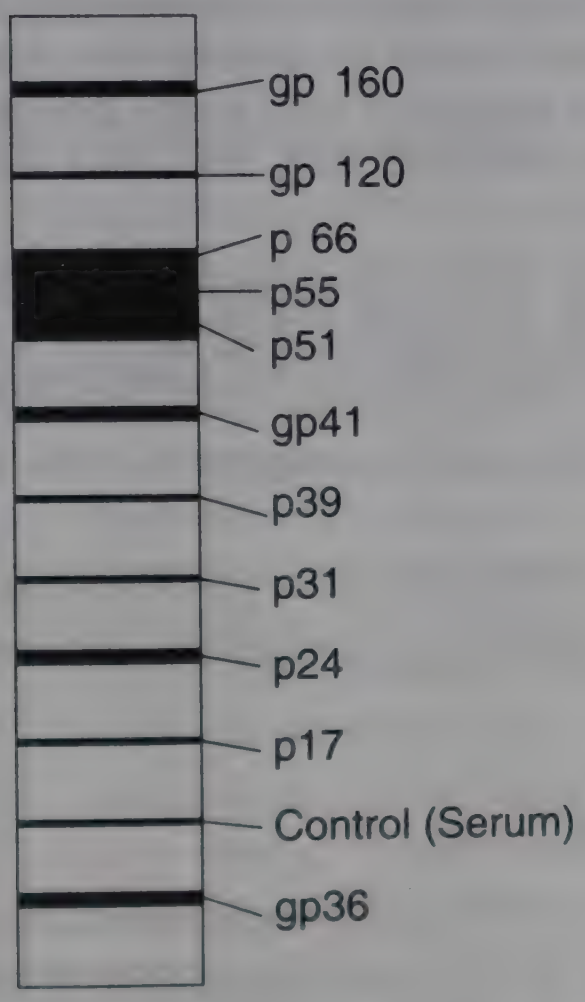


Fig. 7.2 HIV-1 and HIV-2 Western blot

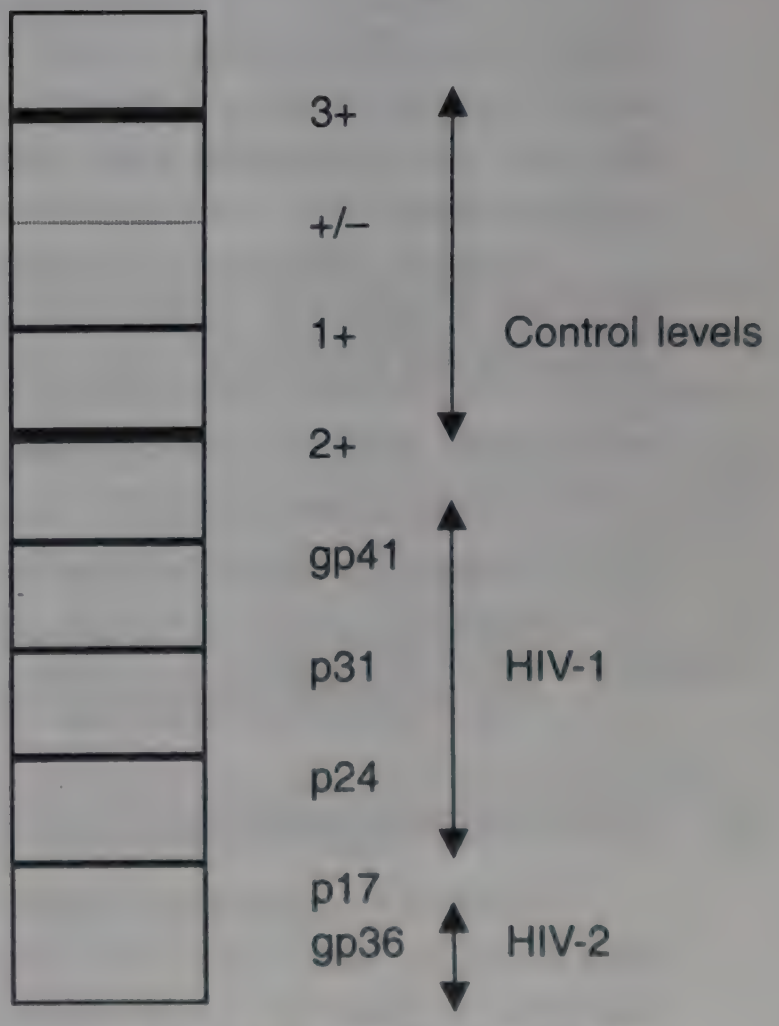


Fig. 7.3 A combination LIA

7.5 PRINCIPLES OF COMBINED ASSAYS FOR HIV-1 AND HIV-2

Here the principle of ELISA remains the same except that antigens of HIV-1 and HIV-2 (recombinant or peptide) are bound to microwells so that antibodies to these antigens (both types) can be detected. Some combined assays use recombinant antigens containing immunodominant regions of the env and gag gene products to make the test extremely sensitive. As a result of this sensitivity, non specific reactions may be seen in some samples. There are combined assays which employ synthetic peptides, which correspond to the highly antigenic and conserved segments of the envelope proteins to HIV-1 and HIV-2 so as to make the tests highly specific and offering the advantage of minimising the incidence of non-specific reactions. For example, in one ELISA format, separate wells are coated with synthetic peptide antigens corresponding to immunodominant regions of the transmembrane of HIV-1 (gp41) or HIV-2 (gp36). The ELISA is performed and the ratio of O.D.s between HIV-2 and HIV-1 wells is determined. If the ratio is >0.2 the specimen is considered to be HIV-2 reactive. If the ratio is <0.5, the specimen is considered to be HIV-1 reactive. Samples that are positive by the combination test should be tested for confirmation by WB assays which could detect both HIV-1 and HIV-2. Line Immunoassays containing recombinant proteins and peptides from HIV-1 and a peptide from HIV-2 are also available nowadays for differential detection of HIV-1 or HIV-2. The results of the tests should be interpreted on the basis of instructions of the manufacturer as well as accepted standard norms of interpretation. It may be necessary to perform sophisticated tests e.g. PCR for confirmation. Nowadays, third generation combination assays have been developed in both microtitre and bead formats. These assays utilise recombinant and/or synthetic peptide antigens to capture antibodies to either HIV-1 or HIV-2 with labelled HIV-1 and HIV-2 antigens being incorporated to bind to antibodies. These antigens have the advantage of being able to detect both IgM and IgG simultaneously, and therefore may offer an increased sensitivity. Dot blot combination assays are also available that have separate dots containing antigens of HIV-1 and HIV-2. With these tests, differentiation of HIV-1 and HIV-2 infections can be accomplished in one go. This saves time and money. Combination tests are more expensive than single detection tests, but are less expensive than using two separate screening tests for HIV-1 and HIV-2, respectively.

Various commercial companies are marketing HIV-1/HIV-2 combination assays. As with HIV-1 assays a variety of formats are available for HIV-2 including beads, microtitre wells and dot blots. There are similarities and differences between the combination assays.

- Most incorporate recombinant and/or synthetic peptide antigens in order to obtain adequate specificity.
- Some use a combination of two synthetic peptides and others as many as four.
- Some use both recombinant and synthetic peptides simultaneously, or viral lysates and synthetic peptides simultaneously.

7.6 LABORATORY DIAGNOSIS OF HIV-2 INFECTION

Most of the principles applied to HIV-1 diagnosis (screening and confirmatory assays) also apply to HIV-2 diagnosis. Accurate diagnosis of HIV-2 infection in the laboratory is essential to know the epidemiology, natural history and pathogenesis of HIV-2 infection. The similarity of HIV-2 to HIV-1 on genetic and antigenic levels has necessitated the development

and implementation of type specific diagnostic assays. Since most HIV-1 kits cross react with HIV-2 antibodies, more sophisticated techniques like synthetic peptide immunoassays and Polymerase Chain Reaction (PCR) have been reliably used for the diagnosis of HIV-2 infection. Patients infected with HIV-2 frequently give positive results with HIV-1 ELISA kits, but remain indeterminant or negative in HIV-1 WB. It is therefore stressed that HIV-1 WB and HIV-2 WB should be done for confirmation of HIV-2 infection when facilities like dot blot hybridisation and PCR are not available.

Although there are similarities in the antigens of HIV-1 and HIV-2, their molecular weight vary slightly. Some tests incorporate certain strains of HIV-2 that contain env antigens of molecular weights similar to those of HIV-1, but antigenically distinct. Most manufacturers of test kits have recently used certain strains of HIV-2 and developed methods to produce some consistency in antigen nomenclature.

7.6.1 Testing for anti-HIV-2 antibodies

Samples showing grey zones and indeterminate results by HIV-1 tests should be subjected to HIV-2 testing. Cross reactions occur with the core proteins (e.g. p24/26 and p55) and pol antigens while the env antigens do not produce cross reactions. Kits incorporating env antigens are more specific for detecting antibodies to this virus.

7.6.1.1 Screening assays

Screening tests for the detection of antibodies to HIV-2 are identical to those of HIV-1, with one exception that the antigens of HIV-2 are used. The screening tests for HIV-1 infection have already been described in chapters 6. Rapid assays i.e dot blot, comb assays are also available and used in the same way as for HIV-1.

7.6.1.2 HIV-2 Confirmation assays

The following HIV-2 confirmatory/supplemental assays are available.

- HIV-2 Western Blot (WB): Several viral lysate HIV-2 WB assays are available. However, concensus confirmatory criteria for HIV-2 positivity have not been developed. Specimens from early seroconverters of HIV-1 patients can display indeterminate HIV-1 WB pattern. The sera from early seroconverters can produce similar results in HIV-2 WB assay. This illustrates the problem of using viral lysate antigens to serologically differentiate between HIV-1 and HIV-2 infections. The general rule is that cross reactions will not occur with the env antigens, at least not to a significant level. Therefore, when HIV-1 WB profile exhibit reactions to gag and/or pol without reactivity to env, HIV-2 infections should be considered and an HIV-2 ELISA/or WB performed (**Fig 7.4**).
- HIV-2 specific ELISA/Rapid/Simple assay using different antigens and different systems. (as in strategy II and strategy III of National HIV Testing Policy).
- Line immunoassays (LIAs).
- Radioimmunoprecipitation assays (RIPA).
- Polymerase Chain Reaction (PCR).

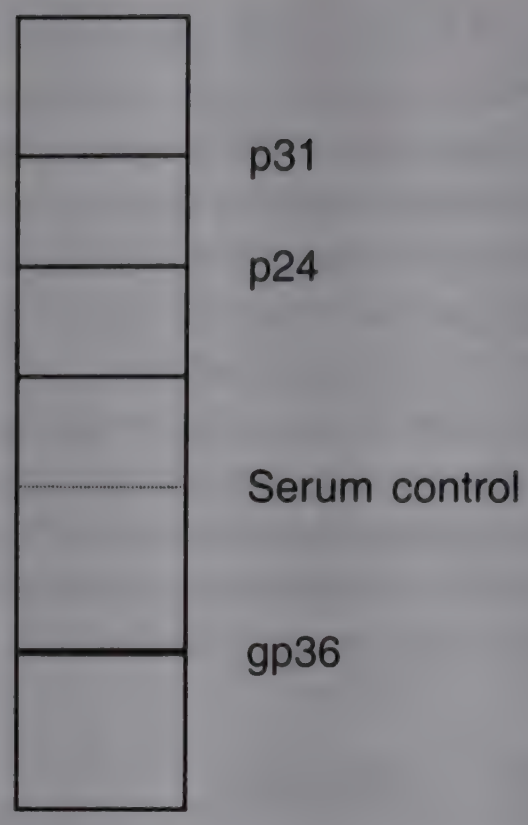


Fig. 7.4 A Typical HIV-2 Seropositive Serum

List of assays available commercially is given in the annexure (WHO approved).

Chapter 8

DIRECT EVIDENCE OF HIV INFECTION

8.1 INTRODUCTION

The diagnosis of infectious diseases can be made with certainty by the direct demonstration of the presence of infecting organisms in the clinical specimens collected from the patient. While isolation of the infecting organism is possible in many infectious diseases, the diagnosis may also be made by detection of nuclear material or antigens specific for the infecting organisms in the clinical specimens.

HIV infection is diagnosed by the presence of anti-HIV antibodies in the blood. However, there are situations where the serology is negative although there is definite evidence of exposure to HIV as seen in patients who are in the window period and in health care workers following accidental exposure to contaminated blood etc. Also children born to HIV infected mothers present dilemma as antibody positivity seen in the infants upto 18 months, may be due to the maternal antibodies in circulation. The diagnosis in these situations may be made by:

- Detection of P24 antigen (discussed in details in Chapter 12)
- Detection of HIV-specific DNA by Polymerase Chain Reaction (PCR)
- Isolation of HIV.

HIV-1 specific DNA amplified in PCR may also be used in Heteroduplex Mobility Assay (HMA) for determining to which subtype the infecting virus belongs. Detection of p24 antigen has been discussed in chapter 12; hence the discussion in this section will restrict to HIV isolation and HIV-PCR including HMA for subtype determination.

8.2 POLYMERASE CHAIN REACTION (PCR) FOR HIV

Serological assays provide a sensitive procedure to screen blood for the presence of antibodies to HIV. Samples found to be positive for HIV antibodies by screening E/R/S tests can be tested by one or two different E/R/S tests (supplemental) based on the prevalence in the population or by the Western Blot test for confirmation. In certain situations however, direct detection of HIV would be needed as seen in the following settings:

Diagnosis:

- Detection of HIV infection in newborns
- Indeterminate Western Blot results
- To monitor viral load levels in HIV infected individuals during anti-retroviral therapy
- To determine HIV status during window period

Research:

- Subtyping of HIV virus
- For cloning genes for use in generating reagents for diagnostic purpose or for preparing vaccines
- For generating chimeric viruses
- For studying functional changes induced by site directed mutagenesis

Because of the sensitivity of the PCR test, it is important that care is taken to avoid cross contamination of samples or carry over of amplified products that can result in false positive results. Since PCR can detect small number of molecules in the sample tested, avoidance of contamination of processed DNA by even minute quantities of target molecules is important to obtain reliable results.

8.2.1 Principle of Polymerase Chain Reaction

PCR helps to find a specific gene sequence amongst an abundance of other DNA. Specific oligonucleotides are chemically synthesised (called primers) which are complimentary to DNA flanking the sequence of interest. Genomic DNA is arranged in the form of a double stranded helix held together by hydrogen bonds. On heating, the double stranded DNA gets denatured to single stranded DNA. On cooling the DNA solution, the primers bind to the single strands of DNA. One primer is designed to anneal to the sense strand and the other primer to the anti-sense strand, with their 3' ends pointing towards each other.

Primers are mixed with a buffered solution of dNTPs, magnesium, thermostable polymerase enzyme and the template DNA. The mixture is then covered with a layer of mineral oil to prevent evaporation and placed in a programmable heating block (thermal cycler). The double stranded DNA template is then denatured by heating to a temperature above its melting point. The temperature is then lowered sufficiently for hybridization between primers and template to occur. The temperature is however, kept high enough to prevent mismatch hybridisation of the primers to similar sequences elsewhere in the genome. High concentration of the primers is maintained to ensure that this reaction is favoured over re-annealing of the complimentary template DNA strands.

The temperature is then raised towards the optimum for the thermostable polymerase enzyme (72°C), which has attached itself to the end of the primer-template duplex. Synthesis proceeds from the 3' end of each primer until the reaction is stopped by heating to the melting point for the second time. The product of this reaction is of indefinite length and is known as long PCR product. This completes the first cycle of PCR.

The second PCR cycle commences with the melting step, followed by primer annealing. Here, the primers anneal not only to the original DNA but also to the newly synthesised strands from the first cycle of PCR. The second cycle repeats the first cycle with respect to the original DNA, but synthesis on the new strands can proceed only as far as the end of the molecule which corresponds to the 5' end of the opposite primer.

After the third cycle of PCR, synthesis directed by the PCR products of the first two cycles will be bound by both ends of the primer sequences and this PCR product will accumulate exponentially with subsequent cycles of amplification. The major product of PCR will thus be of defined length, consisting of the sequence between the primers and includes the primer sequences themselves.

In the HIV PCR reaction, often a nested PCR reaction is used. Here, an aliquot of the PCR product can be re-amplified with a second set of primers which are located at 3' or internal to the original set of primers used for the first round of PCR amplification.

8.2.2 PCR Procedure

8.2.2.1 Sample Preparation for proviral DNA PCR

5 to 10 ml of blood is collected by venepuncture in an EDTA or acid citrate dextrose containing vacutainer tube. 3 ml of Ficoll-Hypaque is pipetted into a conical centrifuge tube. The whole blood collected is carefully layered on top of this Ficoll-Hypaque solution. The tubes are then centrifuged at 2000 rpm for 30 minutes at room temperature in a swing out bucket rotor. The lymphocyte/monocyte fraction appearing between Ficoll-Hypaque layer and plasma layer is transferred to another tube and washed twice with either phosphate buffered saline (PBS pH 7.2-7.4)) or with RPMI 1640. The cells are pelleted by centrifuging at 1500 rpm for 10 minutes. The pelleted cells can be used for DNA extraction or can be resuspended in 90% foetal calf serum (FCS) and 10% DMSO and stored at –70°C or in liquid nitrogen.

8.2.2.2 DNA Extraction

DNA can be extracted from the separated lymphocytes using readymade kits available in the market (e.g. Quiagen, Isoquick). DNA extracted from about 3 million cells is resuspended in 100 µl of water provided in the kit. The DNA can be stored at either –70°C for longterm use or at 4°C if the PCR procedure is to be performed soon after extraction.

8.2.2.3 Primers used for PCR reaction (designed for envelope gene):

First round of PCR

Primer 1

5' TTA GGC ATC TCC TAT GGC AGG AAG AAG CGG 3'

Primer 2

5' TCT TGC CTG GAG CTG TTT GAT GCC CCA GAC 3'

Second round of PCR

Primer 1

5' TGT AAA ACG ACG GCC AGT CTG TTA AAT GGC AGT CTA GC 3'

Primer 2

CAG GAA ACA GCT ATG ACC CAC TTC TCC AAT TGT CCC TCA 3'

8.2.2.4 PCR Reaction

The nested PCR reaction for detection of HIV is carried out in 0.5 ml PCR reaction tubes for each sample, utilising the relevant primers and the patient's DNA sample. In the nested PCR reaction, the first round of PCR is carried out using the patient's DNA sample while the second round of PCR is done using 5 µl of the first round PCR product. PCR is also done for the reference plasmids (obtained from the NIH, Bethesda, USA reagent bank) if heteroduplex mapping assay is to be done for HIV-1 subtyping. Each round of PCR

requires preparation of a master mix composed of the following:

- 10X PCR buffer (10 µl for 100 µl reaction)
- 10 mM $MgCl_2$ (12.5 µl for 100 µl reaction)
- DNTP mixture (200 µM of each dNTP)
- Primer 1 (20 pmole for 100 µl reaction)
- Primer 2 (20 pmole for 100 µl reaction)
- Taq polymerase enzyme (2.5 units for 100 µl reaction)
- dd water (to make up the volume to 100 µl)

The required volume of the master mix is then distributed into the PCR reaction tubes and overlaid with mineral oil. The DNA sample is then added to the PCR reaction tube using barrier tips to avoid cross contamination. The tubes are then placed in the thermal cycler and the PCR reaction carried out. The thermal cycling conditions required are as follows:

3 cycles of the following:

- 94°C for 1 min
- 55°C for 1 min
- 72°C for 1 minute

followed by 32 cycles of the following:

- 94°C for 15 sec
- 55°C for 45 sec
- 72°C for 1 min.

After this, the PCR reaction is maintained at 72°C for 5 minutes and then cooled to 4°C. After completion, the amplified PCR products are run on agarose gel to detect the presence of the PCR products. 10 µl of each second round PCR product of a nested PCR reaction is mixed with 2 µl of a 6X bromophenol blue/xylene cyanol dye mix and run on a 1% agarose gel containing ethidium bromide (30 µl of a 10 mg/ml solution added to 50 ml of 1% agarose solution made in 1X TAE buffer). Simultaneously, a DNA marker should be run with every gel to detect the size of the PCR product (1 Kb DNA ladder could be used). After running the gel on a horizontal gel electrophoresis apparatus, the gel is transferred onto a UV transilluminator and a picture of the gel taken using a suitable gel documentation system. Presence of the PCR product of the required size (as shown by comparison of the PCR product with the DNA bands seen in the molecular weight marker lane) indicates a positive PCR reaction. With the above mentioned primers, a PCR product of 700 base pair size should be detected.

8.2.2.5 Precautions to be followed for PCR reaction:

- All blood samples must be handled carefully to avoid possibility of transmission of HIV. Lab coats and gloves should be used while handling the samples and PCR products. Biosafety laminar flow hoods should be used to prevent cross contamination as well as for biosafety precaution.

- Use of a dedicated area that is free of PCR product for the preparation of the PCR reaction. Amplified products are to be kept away from the sample processing area as far as possible.
- Use of barrier tips or positive displacement pipettes for dispensing of samples.
- Reagents should be stored as small aliquots where possible
- Preparation of master mixes to minimize handling of reagents

Fig. 8.1 depicts the principle of PCR procedure and **Fig. 8.2** shows the bands obtained after gel electrophoresis of env PCR products (700 bp) run with λ/Hind III marker

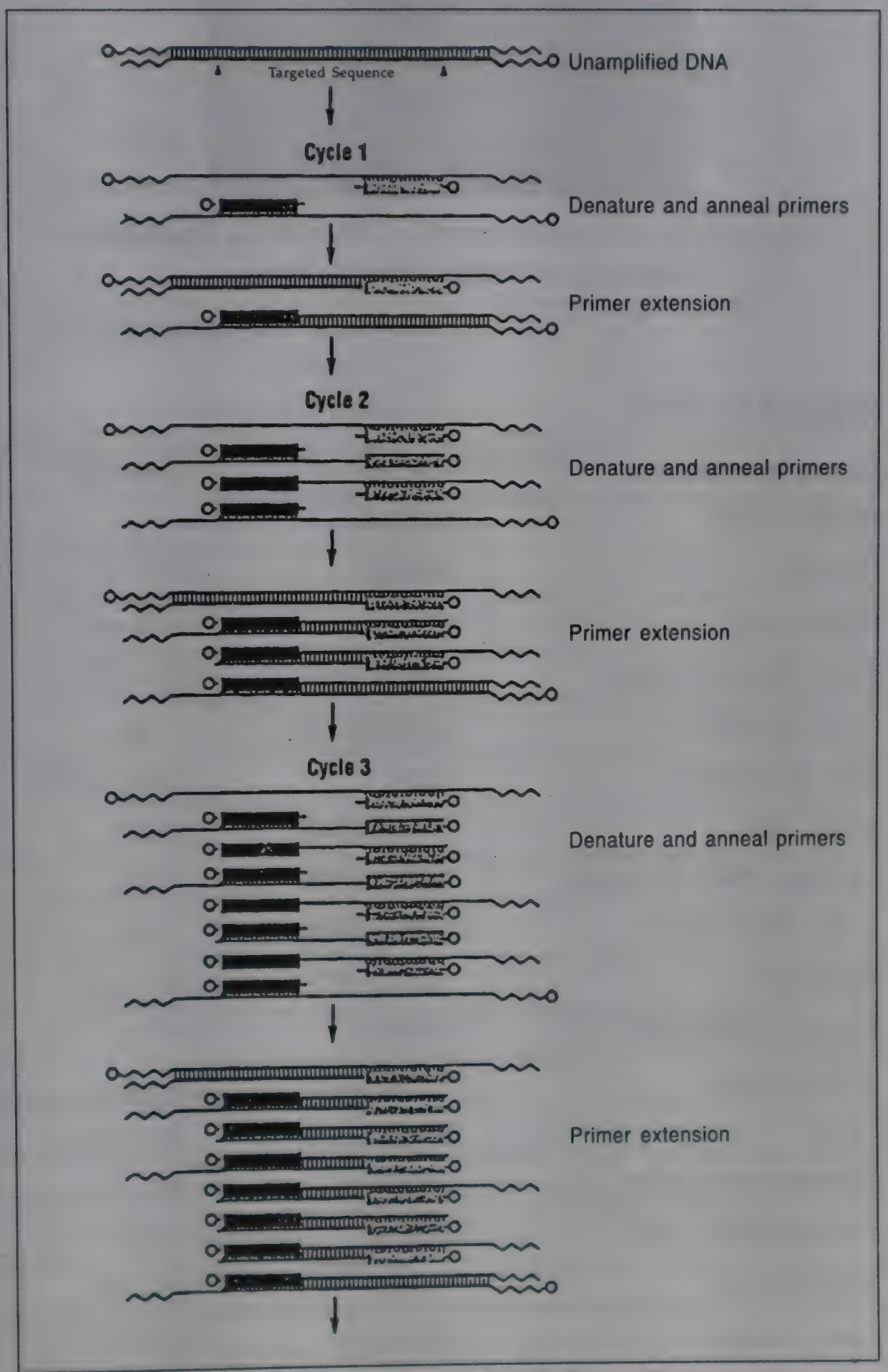


Fig. 8.1 Principle of PCR Procedure

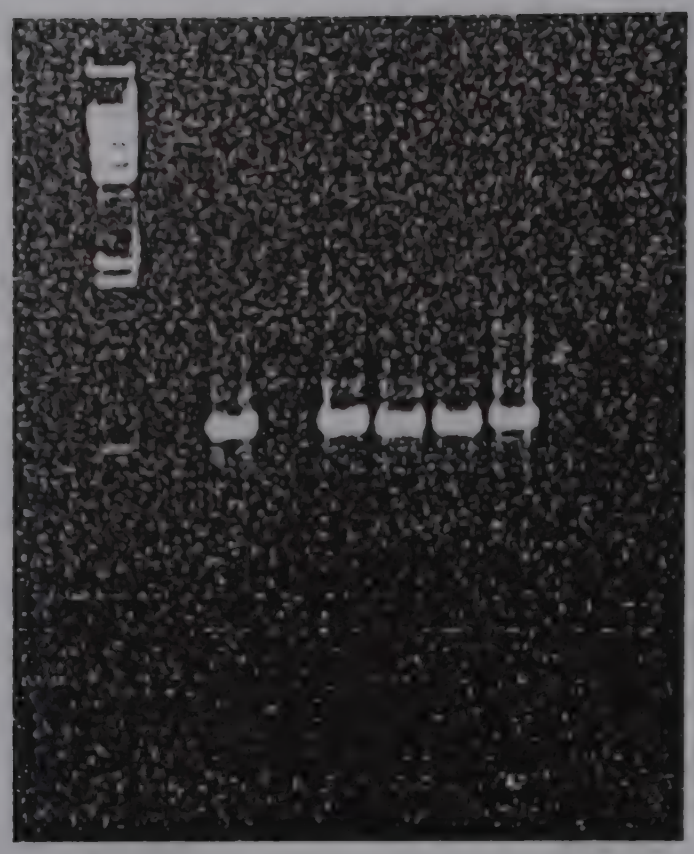


Fig. 8.2 Gel Electrophoresis of ENV PCR Products (700 bp) run with λ/HindIII Marker

8.2.3 Reagents required for PCR

Sr.No.	Name of chemical	Cat.No.	Quantity	Price
01.	RPMI 1640	R6504	10 L	$ 13.80
02.	DMSO	D2650	100 ml	$ 76.60
03.	Histopaque	H8889	500 ml	$ 76.50
04.	Fetal Bovine Serum	16000-044	500 ml	$210.00
05.	QIAamp blood kit	GX2670QN	1 No.	Rs. 7226/-
06.	Taq. Polymerase	LB9	1000 Units	Rs. 8000/-
07.	dNTP set	GX5341MB	1 set	Rs. 7373/-
08.	Lambda Hind III digest	GX5251MB	1 vial	Rs. 2396/-
09.	1 Kb DNA ladder	GX5249MB	1 vial	Rs. 4792/-
10.	Agarose	15510-027	500 gm	$ 364.00
11.	Ethidium Bromide	E1510	10 ml	$ 30.10
12.	Trizma base	T1503	5 Kg	$ 267.30
13.	EDTA	E5134	500 gm	$ 66.80
14.	Glacial acetic acid	11005	500 ml	Rs. 104/-
15.	Bromophenol blue	B5525	5 gm	$ 13.50
16.	Isopropanol	13825	500 ml	Rs. 100/-
17.	Ethyl Alcohol	10107	500 ml	Rs. 575/-
18.	Xylene cyanol	X4126	10 gm	$ 22.10

The reagents listed above are those being used at National AIDS Research Institute (NARI), Pune. The company's name has been deliberately withheld to avoid bias. Molecular biology standard reagents can be purchased from any company dealing in such chemicals and reagents.

8.2.4 Equipment required for PCR

Sr.No.	Name of Equipment
01.	Horizontal gel electrophoresis apparatus
02.	Power supply Model 250 (for PCR) Model 500 (for HMA)
03.	Vertical Gel Electrophoresis apparatus (for HMA)
04.	UV Transilluminator
05.	Gel documentation system
06.	PCR tubes 1.5 ml 0.5 ml
07.	Micropipettes P 20 P 200 P 1000
08.	Laminar flow (2'x2'x2' for reagents) (4'x2'x2' for samples)
09.	Heat block
10.	Eppendorf centrifuge
11.	Programmable Thermal Controller (PCR machine)
12.	UPS (1 KVA) with 4 batteries
13.	Vortex mixer
14.	Water bath
15.	Microwave oven
16.	Pipette aid with charger
17.	Refrigerater

8.3 Heteroduplex Mobility Assay (Hma)

If the PCR products are to be used for subtyping of the HIV-1 virus in the sample for epidemiological reasons, the heteroduplex mobility assay could be carried out. Heteroduplexes are formed by melting amplified PCR product from unknown patient's sample with amplified PCR product from reference strains and analysed on polyacrylamide gel electrophoresis. Heteroduplexes formed between the unknown sample and the most closely related sequences exhibit the fastest mobilities on polyacrylamide gel electrophoresis. 5 µl of the second round PCR product of patient's sample is mixed with 5 µl of PCR product of different reference

plasmids in different tubes together with 1.1 µl of annealing buffer in each tube and heated in a thermal cycler for 2 minutes at 94°C and then placed on wet ice. In one of the tubes, 10 µl of the patient's PCR product is taken together with annealing buffer without the addition of reference plasmid PCR product. The heteroduplex reaction is mixed with 3 µl of 5X loading dye and then loaded onto a 5% non-denaturing polyacrylamide gel and run at constant current. After running the samples for adequate duration (depending on the size of the PCR product), the electrophoresis is terminated and the acrylamide gel is placed in ethidium bromide containing buffer for about an hour. Subsequently, the acrylamide gel is placed on a UV transilluminator and photographed using suitable filter lenses. Depending on the pattern of the bands observed, the subtype of HIV-1 in the DNA sample can be determined.

8.3.1 Reagents required for Hma

S.No	Name of chemical	Make	Cat.No.	Quantity	Price
1.	Acrylamide (for HMA)	Gibco BRL	15512-023	500 gm	$ 103.00
2.	Bis-acrylamide (for HMA)	Gibco BRL	15516-024	100 gm	$ 64.00
3.	Boric acid (for HMA)	Sigma	B7660	1 Kg	$ 49.90
4.	Ammonium per sulfate (for HMA)	Sigma	A9164	25 gm	$ 13.70
5.	TEMED (for HMA)	Sigma	T8133	50 ml	$ 10.40

8.4 ISOLATION OF HIV

HIV may be isolated from the blood and other body fluids. Isolation of HIV is a specialized procedure requiring at least P2+ containment facility and high degree of expertise. HIV isolation has therefore remained mostly as a tool for research. HIV is isolated from the peripheral blood mononuclear cells (PBMC) or plasma and other body fluids. The activated CD4+ cells are susceptible for HIV infection. Hence autologous or heterologous PBMCs activated with mitogen Phytohaemagglutinin (PHA) are cultured with the infectious material. The cultures are maintained at 37°C in 5% CO_2 atmosphere for up to 28 days. The cultures are fed with fresh activated PBMCs at regular intervals. The presence of virus in the culture supernatant is detected either by demonstration of the presence of p24 antigen or enzyme reverse transcriptase. The infected cells may also demonstrate syncitia in culture. Virus infected cells may also be detected by HIV-specific immunofluorescent assay.

The two methods normally used for virus isolation are direct method or co-culture method. In the direct method, PBMCs from the patient are cultured in vitro in presence of PHA. In the co-culture method PBMCs from heterologous HIV uninfected donor are stimulated with PHA, and after 48-72 hours the stimulated cells are cultured along with the PBMCs from the patient. In our experience, co-culture method gives greater success in virus isolation.

Chapter 9

GUIDELINES FOR HIV TESTING

9.1 INTRODUCTION

The HIV/AIDS epidemic has raised a number of issues particularly in the health care system and one of the very important issues has been the HIV test itself. HIV testing has generated interest not only in the scientific community but also amongst the general public. The various technical, ethical and legal issues that invariably accompany the HIV testing have led the countries the world over to develop their own HIV testing policies and guidelines. The development of tests to detect infection with HIV has made it possible to determine the prevalence of HIV infection and to monitor trends of the infection in populations. Though this information is of great value in designing, implementing and monitoring public health programs for the prevention and control, however, testing of any population for HIV requires careful consideration of a number of points relating to logistics, laboratory, operational, legal and ethical. These aspects should be kept in mind while prescribing any HIV test.

9.2 OBJECTIVES OF HIV TESTING

The early detection of HIV infection differs from similar detection of most other infectious diseases on account of the following reasons:

- Due to prolonged asymptomatic stage of infection, one remains fully active and demands an appropriate intervention which maintains the lifestyle and dignity of the individual.
- HIV infection is believed to be invariably fatal irrespective of the best possible treatment.
- HIV infection and AIDS are still associated with high degree of discrimination and stigmatisation.
- The implications of a positive test go well beyond those related to physical and mental health of the individual being tested.

Thus, any HIV testing that is done has any of the four following objectives:

- To monitor the trends of HIV infection in a population or subgroup for facilitation of intervention (surveillance: unlinked and anonymous).
- To test donated blood or donors of organs or tissues for ensuring safety of the recipients (transfusion safety and donation safety).
- To identify an individual with HIV infection for diagnosis (with AIDS indicator diseases) or voluntary testing purposes (asymptomatic or AIDS cases).
- Research.

9.3 HIV TESTING STRATEGY

Often the separate objectives mentioned above cannot be met by a single testing

strategy. Different objectives require separate testing procedures and the choice of tests (discussed in detail in chapters 6). Based on relevant consideration (chapter 6) different procedures and strategies of testing are adopted.
The different procedures for testing are:

9.3.1 Unlinked anonymous testing

Such type of screening or testing is not directed to the individual, but has as its objective, the public health surveillane of HIV infection. It is an epidemiological method for measuring HIV prevalence in a selected population with the minimum of participation bias. By minimising participation bias, unlinked anonymous screening offers a distinct epidemiological advantage over mandatory or voluntary testing. Unlinked anonymous testing involves use of blood already collected for other purposes; therefore, the effect of selection bias will remain and will depend upon time, location and other details of blood collection.

9.3.2 Voluntary confidential testing

Testing is often done for diagnostic purposes. Here it is important that the issues related to confidentiality receive great attention. Since this method is based on voluntary HIV testing or testing for diagnosis of HIV/AIDS cases, it is imperative to respect the individual's need to maintain confidentiality. By maintaining confidentiality, it will not only instill faith in the individual about the health care system in the community but also encourage more and more people practicing risk behaviour to come forward for an HIV test.

9.3.3 Mandatory testing

When testing is done without the consent of the patient and data could be linked to identify the person it is called "mandatory testing". Mandatory testing is recommended only for screening donors of semen, organs or tissues in order to prevent transmission of HIV to the recipient of the biological products.

The choice of tests is also based on the different objective of HIV testing. The test that are adopted are the ELISA, Rapid or Simple, clubbed together as 'E/R/S'. One E/R/S denotes test done on one single antigen preparation; two is when all positive samples on first antigen test is repeated on a second antigen preparation and three is when this test is repeated for a third time using a different antigen system. For transfusion safety purposes one E/R/S is used; for surveillance and diagnosis of full blown AIDS cases two E/R/S are used and for asymptomatic individuals three E/R/S are used.

9.4 GENERAL PRINCIPLES OF HIV TESTING

Testing policy in general should consider the following points:

- It should be part of the overall comprehensive and preventive program.
- Testing should be technically sound and appropriate.
- Test procedure must be appropriate to the field situation.
- Testing procedure must be cost effective.

- Laboratory procedure must be monitored for ensuring quality.

9.5 HIV TESTING IN HEALTH CARE SETTINGS

The fear and apprehension that exists among health care workers in managing HIV infected individuals and AIDS patients is largely due to the minimal risk that exists of HIV transmission due to a needle stick or other sharp injury. Thus the demand for mandatory HIV testing of patients admitted in hospitals or undergoing surgery etc. This demand is neither rational nor appropriate. A mandatory HIV test is no substitute for **Universal Precautions** that need to be adopted for every patient in a hospital or any other health care setting. On the other hand testing without **explicit consent** of the patient has been proved to be counterproductive in the long run. In the control of the HIV epidemic such testing can drive the target people underground and make it more difficult for launching interventions.

The **national testing policy** reiterates the following:

- No individual should be made to undergo a mandatory testing for HIV.
- No mandatory HIV testing should be imposed as a precondition for employment or for providing health care services and facilities.
- Any HIV testing must be accompanied by a pretest and post test counselling services.

List of voluntary blood testing centers and references centers (NACO) are given in Annexure 9.

Chapter 9 - Annexure I

HIV testing facilities have been classified into zonal blood testing centres (ZBTCs) which screen the donated units of blood, surveillance centres which carry out HIV testing for surveillance, voluntary blood testing centres where HIV testing for diagnosis and identification of the individual is undertaken with pre and post test counselling and national reference centres which undertake all the above activities along with the jobs defined under the centres. List of National Reference Centes and the Voluntary and Surveillance Testing Sites is placed below for ready reference.

List of HIV Reference Centres

1. National Institute of Communicable Diseases, Delhi.
2. All India Institute of Medical Sciences, New Delhi.
3. Indian Institute of Immunohaematology, Mumabi.
4. National Institute of Cholera and Enteric Diseases, Calcutta.
5. School of Tropical Medicine, Calcutta.
6. Madras Medical College, Chennai.
7. Naional AIDS Research Institute (NARI), Pune.
8. Regional Medical College, Imphal.
9. Christian Medical College, Vellore.

The reference centres are entrusted with the responsibility of carrying out confirmatory test. They are also responsible for diagnosis, quality control of HIV kits, guidelines for HIV testing, training in HIV testing and any other activity which may be necessary for standardization of HIV testing. These centres also undertake voluntary blood testing with pre and post test counselling. Also provide training for practice of "Biosafety Precautions" and "Quality Assurance Systems."

List of surveillance and voluntary blood testing centres sanctioned upto 1998

Sanctioned in 1998	Existing
1. ANDHRA PRADESH	
i Andhra Medical College, Vishakhapatnam.	x Deptt. of Microbiology, Osmania Medical College, Hyderabad.
ii Rangaraya Medical College, Kakinada.	xi Deptt. of Microbiology, S.V. Medical College, Tirupati.
iii. Guntur Medical College, Guntur.	
iv. Siddhastha Medical College, Vijayawada.	xii Deptt. of Microbiology, Andhra Medical College, Vishakhapatnam.
v. Osmania Medical College, Hyderabad.	
vi Gandhi Medical College, Secunderabad.	xiii Surveillance Centre, Instt. of Prev. Medicine, Hyderabad
vii Kaktiya Medical College, Warrangal.	
viii Kurnool Medical College, Kurnool.	xiv Surveillance Centre, Indian Naval Ship Hospital, Kalyani, Vishakhapatnam.
ix S.V.Medical College, Tirupati	

2. ASSAM

i Guwahati Medical Cellege, Guwahati.
ii Silchar Medical College, Silchar.
iii Assam Medical College, Dibrugarh.
iv Deptt. of Microbiology, Guwahati Medical College Guwahati

3. BIHAR

i Darbhanga Medical College, Leharia Sarai.
ii S.K. Medical College, Muzzafarpur.
iii Patna Medical College, Patna
iv Rajendra Medical College, Ranchi.
v M.G.M. Medical College, Jamshedpur.
vi Patliputra Medical College, Dhanbad.
vii Medical College, Bhagalpur.
viii Magadh Medical College, Gaya.
ix Nalanda Medical College, Patna.
x Rajendra Memorial Research Institute, Patna

4. DELHI

i A.I.I.M.S., New Delhi.
ii M.A.M.C., New Delhi.
iii U.C.M.S., New Delhi.
iv Lady Hardinge Medical College, New Delhi.
v Deptt. of Microbiology, University College of Medical Sciences, Shahdara, Delhi.
vi Deptt. of Microbiology, Maulana Azad Medical College, New Delhi
vii Surveillance Centre, Armed Forces Command Hospital, Delhi Cantt.

5. GOA

i Goa Medical College, Goa.
ii Deptt. of Microbiology, Goa Medical College, Panaji

6. GUJARAT

i B.J. Medical College, Ahmedabad.
ii Municipal Medical College, Ahmedabad.
iii Medical College, Baroda.
iv M.P. Shah Medical College, Jamnagar.
v Govt. Medical College, Surat.
vi Deptt. of Microbiology, B.J. Medical College, Ahmedabad

7. HARYANA

i Govt. Medical College, Rohtak.
ii Deptt. of Microbiology, Medical College Rohtak.

8. HIMACHAL PRADESH

i I.G.M.C. Shimla.
ii Deptt. of Microbiology, Indira Gandhi Medical College Shimla.

9. JAMMU and KASHMIR

i Govt. Medical College, Srinagar.
ii Govt. Medical College, Jammu.
iii Sher-e-Kashmir Institute of Medical Sciences, Srinagar.
iv Department of Immunopathology, Sher-e-Kashmir Instt. of Medical Sciences, Srinagar.
v Deptt. of Microbiology, Govt. Medical College Jammu.

10. KARNATAKA

i Mysore Medical College, Mysore.
ii Bangalore Medical College, Bangalore.
iii Karnataka Medical College, Hubli.
iv Medical College, Bellary.
v Medical College, Mangalore.
vi Deptt. of Microbiology, Bangalore Medical College, Bangalore.
vii Deptt. of Microbiology, Kasturba Medical College, Manipur.

11. KERALA

i Medical College, Trivandrum.
ii T.D. Medical College, Allepey.
iii Medical College, Calicut.
iv Medical College, Trichur.
v Medical College, Kottayam.
vi Deptt. of Microbiology, Medical College, Trivandrum.
vii Surveillance Centre, Indian Naval Ship Hospital, Cochin.

12. MADHYA PRADESH

i Govt. Medical College, Jabalpur.
ii G. R. Medical College, Gwalior.
iii M.G.M. Medical College, Indore.
iv Gandhi Medical College, Bhopal.
v S.S. Medical College, Rewa
vi Pt. J.L.N. Medical College, Raipur.
vii Deptt. of Pathology, Gandhi Medical College, Bhopal.
viii Regional Medical Research Centre for Tribal Health, Jabalpur.
ix Choitram Hospital and Research Centre, Indore.

13. MAHARASHTRA

i Grant Medical College, Mumbai.
ii Seth. G.S. Medical College, Mumbai.
iii T. N. Medical College, Mumbai.
iv L.T.M. Medical College, Mumbai.
v B.J. Medical College, Pune
vi A.F.M.C., Pune.
vii Miraj Medical College, Sholapur.
viii Dr. V. M. Medical College, Sholapur.
ix Govt. Medical College, Aurangabad.
x S.R.T.R. Medical College, Ambajogari.
xi Medical College, Nagpur.
xii Indira Gandhi Medical College, Nagpur.
xiii Govt. Medical College, Nanded.
xiv Deptt. of Microbiology, Seth G.S. Medical College, Mumbai.
xv Deptt. of Microbiology, J.J. Hospital, Mumbai
xvi Sion Hospital, Mumbai
xvii B.Y.N. Nair Hospita, Mumbai
xviii Rajabari Hospital, Ghatkopar, Mumbai.
xix Deptt. of Microbiology, B.J. Medical College, Pune.
xx Deptt. of Microbiology, Govt. Medical College, Nagpur.
xxi Surveillance Centre, Civil Hospital, Kolhapur

xxii Surveillance Centre,
District Hospital, Chandrapur.
xxiii Deptt. of Microbiology,
Govt. Medical College, Miraj.
xxiv Surveillance Centre, Indian Naval Ship
Hospital, Ashwini, Mumbai.
xxv Deptt. of Microbiology,
Armed Forces Medical College, Pune.

14. ORISSA

i S.C.B. Medical College, Cuttack.
ii V.S.S. Medical College, Buria.
iii M.K.C.G. Medical College,
Berhampur.
iv Deptt. of Microbiology,
S.C.B. Medical College, Cuttack
v Surveillance Centre, Regional Medical
Research Centre, Bhubneshwar.

15. PONDICHERY

i J.I.P.M.E.R.
ii Surveillance Centre,
Govt. General Hospital, Pondicherry.
iii Deptt. of Microbiology,
JIPMER, Pondicherry.

16. PUNJAB

i Medical College, Amritsar.
ii Medical College, Patiala.
iii Medical College, Faridkot.
iv Deptt. of Microbiology,
Govt. Medical College, Amritsar.

17. RAJASTHAN

i S.M.S. Medical College, Jaipur.
ii S.P. Medical College, Bikaner.
iii R.N.T. Medical College, Udaipur.
iv Dr. S.N. Medical College, Udaipur.
v J.L.N. Medical College, Ajmer.
vi Deptt. of Microbiology,
S.M.S. Hospital, Jaipur.

18. TAMIL NADU

i Govt. Medical College, Chennai
ii Stanley Medical College, Chennai
iii Kilpauk Medical College, Chennai
iv Sri Ramachandra Medical College
and Research Institute, Chennai.
v Thanjabur Medical College, Thanjabur.
vi Medical College, Coimbatore.
vii Madurai Medical College, Madurai
viii Tiruneveli Medical College, Tiruneveli.
ix Medical College, Chingleput.
x Deptt. of Microbiology, Instt. of Child Health
and Hospital for Children, Madras.
xi Deptt. of Microbiology,
Madurai Medical College, Madurai.
xii Surveillance Centre,
Medical College, Chennai.

19. UTTAR PRADESH

i S.N. Medical College, Agra.
ii M.L.N. Medical College, Allahabad.
iii J.N. Medical College, Aligarh.
iv Institute of Medical Science, BHU, Varanasi.
v G.S.V.M. Medical College, Kanpur.
vi M.I.B. Medical College, Lucknow.
vii K.G. Medical College, Lucknow.
viii B.R.D. Medical College, Gorakhpur.
ix Deptt. of Microbiology, K.G. Medial College, Lucknow.
x Surveillance Centre, Central JALMA Instt. for Leprosy, Agra
xi Deptt. of Microbiology, Instt. of Medical Sciences, Varanasi.
xii Deptt. of Microbiology, J.L.N. Medical College, Aligarh.
xiii Surveillance Centre, Kamla Nehru College, Allahabad.

20. WEST BENGAL

i Govt. Medical College, Calcutta.
ii R.G. Kar Medical College, Calcutta.
iii N.R.S. Medical College, Calcutta.
iv National Medical College, Calcutta.
v B.S. Medical College, Bankura.
vi North Bengal Medical College, Siliguri.
vii Surveillance Centre, National Institute of Hygiene and Public Health, Calcutta.

21. CHANDIGARH

i P.G.I. of Medical Education and Research, Chandigarh.
ii Deptt. of Immunopathology, PGI, Chandigarh.

22. MANIPUR

i Regional Medical College, Imphal.
ii Surveillance Centre, J.N. Hospital, Imphal.

23. MEGHALAYA

i Surveillance Centre, Civil Hospital, Shillong.

24. MIZORAM

i Surveillance Centre, Civil Hospital, Aizwal.

25. NAGALAND

i Surveillance Centre, Naga Hospital, Kohima.
ii Surveillance Centre, Distt. Hospital, Dimapur.

26. ARUNACHAL PRADESH

i Surveillance Centre, Distt. Hospital, Itanagar.

27. SIKKIM

i Surveillance Centre,
S.T.N.M. Hospital, Gangtok.

28. TRIPURA

i Surveillance Centre,
Distt. Hospital, Agartala.

29. ANDAMAN AND NICOBAR ISLANDS

i Surveillance Centre,
G.B. Hospital, Port Blair.

30. LAKSHADWEEP

i Surveillance Centre,
Govt. Hospital, Kavaratti.

31. DADRA AND NAGAR HAVELI

32. DAMAN AND DIU

Chapter 10

QUALITY ASSURANCE PROGRAMME

10.1 NECESSITY AND IMPORTANCE

The diagnostic tests to detect antibodies to HIV have sensitivity and specificity which are not absolute. In all these tests we have false negative results as well as false positive results which are inherent and cannot be avoided. The percentage of false positive results will increase as the prevalence rate of persons with HIV antibodies in a population decreases. *These two problems are compounded by the fact that during screening programmes, laboratories will rarely be able to perform to the level of accuracy that the tests are technically capable of achieving.* Thus, the validity of diagnostic test results is dependent to a very large extent on the quality of the technical conditions under which the tests are performed. Meaning thereby, consistent production of reliable results requires a stringent overall assurance programme which would control technical conditions before, during and after each assay.

The Quality Assurance Programme ensures that the final results reported by the laboratory are correct, reliable and accurate as far as possible. This programme oversees reporting results in a timely manner and to the appropriate individual. It also ensures use of the most reliable tests for the diagnosis of HIV infection. It is dependent on a good **Quality Control Programme** and its efficacy may be verified by a good **Quality Assessment Programme**.

- **Quality control programme**

  This programme includes measures which are introduced during each assay to verify the validity and reliability of the test. However, this does not indicate that the results generated are accurate (which, indeed, is the characteristic of the test). It also does not indicate that the results have been reported timely, properly and correctly.

- **External quality assessment programme**

  This is a means to determine the quality of results. It is an external evaluation of a laboratory performance by incorporating proficiency panels as the means to evaluation. For a laboratory to be considered a respected testing facility, it must be a laboratory that can always produce accurate, reliable and reproducible results.

10.2 GUIDELINES TO IMPROVE QUALITY OF TESTING

i) Condition of the specimens

All specimens must be inspected at the time of receiving and also before testing to ensure that they are suitable. Use of lipaemic, haemolysed and contaminated sera should be avoided. If they have to be used, the reporting officer should mention on the report that the result of the test may not be valid because of the condition of the serum samples. A new specimen should be requested for repeat testing.

All specimens should be properly labelled before acceptance. The label should include following information: a) name of the patient, b) collection date, c) patient's identification number if applicable. Each specimen must be accompanied with a test request form which should include age and sex of the patient, name of the physician requesting the investigation, risk group of the patient, reason for the investigation in addition to the information given on the label. In case of unlinked anonymous testing, only code number and collection date may be mentioned on the label as well as on the request form.

If the serum or plasma sample is frozen before testing, it is essential that after thawing at room temperature 37°C, the sample should be inspected for any clot or floating particles. The sample should be clarified before testing. The sample should be well-mixed before testing.

Presently, all the test kits distributed by the NACO, are meant for testing serum or plasma only. Therefore, these kits may not reliably detect presence of antibodies in other body fluids.

ii) Quality of kits and equipments

The test kits must be used within the expiration date stated on the kit to ensure valid results.

Every batch of the kit should be tested and certified for its efficacy (sensitivity and specificity) before distribution (see chapter 11).

ELISA reader, washers, incubators, pipettes should be checked regularly for their optimal performance. They need to be calibrated at least annually.

iii) Controls used in the tests

Each test run requires a set of controls to validate the results. These controls must be treated in the same manner as unknown samples. They are run simultaneously and under the same conditions as the unknown samples. Upon completion of the test, the results of the controls and the samples are examined using the same criteria for interpretation. The assay is valid and the results are reliable when the controls produce acceptable results.

There are two types of controls which must be included in each run:

a) Internal controls

These controls (positive and negative) are included in each HIV-test kit by the manufacturer and are to be included in each test run. These are essential for quality control measures for each run. They are intended for use with the same lot number of the kit in which they have been packed. Internal kit controls are generally adjusted by the manufacturer so that an expected range of values are obtained with each lot of kits. To avoid considerable fluctuations in the OD values due to variable coating of the antigen on the solid surface during manufacturing, the manufacturer, artificially stabilizes kit controls to give the same values as the previous lot.

b) External controls

These should be included with each test-run to monitor consistent performance and lot to lot variation which cannot be detected using internal controls for the reasons

mentioned above. These controls are made from pooled test kit controls or made from pooled sera from HIV-positive or negative individuals in each laboratory (in-house controls). However, for monitoring the performance of other laboratories, the serum samples for external controls are drawn in sufficient quantities to last for at least 12 months (chapter 11).

The most important external control to include is a borderline reactor. This control would indicate any minor change specially around cut off value because such changes would effect the results of unknown samples with OD values near the cut off. It may take several months to establish ranges for external controls. Nonetheless, this type of control system can be very effective in helping to identify potential problems and inaccuracies in the laboratory setting.

c) Standardizaiton of the quality of external controls

Reproducibility and quality of internal and external controls must be standardized by **intra-run reproducibility** and **inter-run reproducibility**. The control samples (either internal or external) are tested at least three times on the same test-run. This will indicate intra-run reproducibility. This exercise is then carried out on test-runs on three consecutive days to determine inter-run reproducibility. By either of these methods variations should not exceed 10%. These serum samples may also be evaluated at the National Reference Centers.

iv) Interpretation of data

The test kit inserts carry instructions to establish range of internal controls and to define the outliers. Since the external controls are included in each test run to monitor consistent performance of the test kit, it is necessary to determine the limit of acceptability statistically by calculating arithmetic mean, standard deviation and error, coefficient of variation etc. However, the values for the internal controls, external controls and the cut off can be monitored easily by quality control graphs. This is because the graphic presentation of the control values over time will make subtle changes in controls more easily discernible.

An accurate method of graphically representing the values of control of each test run is to plot OD/cut-off (CO) ratio on Y-axis. In this method the control values are expressed relative to the cut-off value. This is important as the OD as well as CO values will change slightly between test-runs. Therefore, the controls should be compared with the respective calculated CO value.

Two types of changes can be observed in the quality control graphs. These are.

a) Shifts

When control values of six consecutive test runs fall on one side of the mean, it is called shift. This indicates a major change in the test-performance due to i) switching to a new lot of kits, ii) new reagents, iii) changes in incubation temperature, iv) change of pipettes, v) a new technician, etc.

b) Trends

When control values of six consecutive tests are distributed in one general direction, it is called trend. This is generally due to i) deterioration of reagents, and ii) a routinely used pipette slowly losing its calibration.

v) Calculation of grey-zone reactors

During routine testing, many samples may have slightly elevated OD values just above CO value which may suggest presence of low antibody activity. Such samples are called grey-zone reactors or borderline reactors. This reactivity may be due to a) early seroconversion; b) very low antibody reactivity present in the serum; e) false positive reactivity. Therefore, it is very essential to repeat any HIV reactive result having OD values greater than or equal to the cut-off value. Any repeatedly positive result is then validated by a more specific supplemental test like Western blot.

Sometimes, due to a technical error a serum with low antibody reactivity may give an OD value just below the cut-off value. Therefore, many laboratories advocate a repeat HIV test on all the samples which give an OD value of 10% below the cut-off. Although this is very arbitrary, this approach increases the chance of finding some early HIV seroconverison.

vi) Accuracy of Enzyme Immunoassay (EIA)

Since no assay can be 100% sensitive and 100% specific, it is likely to give false negative as well as false positive results.

Any EIA may be false negative under following conditions:

a) Biological Conditions

- The test may be false negative during early stages of HIV infection. This period generally varies from 2-24 weeks but may be as long as 42 months. the false negative rate varies from 16% to 50% during this period. (Farzadegan *et al., J. Clin. Microbiol* **27**: 1882, 1989)
- In some patients there is an early production of antibodies followed by a cessation of antibody production and disappearance of antibodies from blood. This may be due to sequestration of the virus.

b) Technical conditions

- The test kit generally has sensitivity ranging from 99.4% to nearly 100%. However, if performed under less optimal conditions the sensitivity may drop considerably.

The EIA may be false positive under following conditions:

a) Biological conditions:

- Certain conditions increase false positivity rates of ELISA. These are chronic alcoholism, parenteral drug abuse, haemodialysis, multiple pregnancies and multiple blood transfusions.
- False positivity rises if the prevalence of HIV antibodies in the population decreases.

b) Technical conditions:

- False positive rates vary from one ELISA kit to another. Even the same type of kit may show a significant variation in specificity from batch to batch.
- Human error, e.g., specimen mixing may account for false positivity.
- Laboratories with less than optimum performance also account for higher false positivity rates.

vii) Reporting of results

Unlike any other test report, the HIV-test reports must be handled with care. Since HIV infected individuals may face social stigma, improper reporting may sometimes lead to emotional breakdown of an individual or even suicides. Therefore, before reporting the result of HIV-antibody test it must be borne in mind that:

a) The results of any screening test (ERS) are only presumptive and should not be reported. These must be validated by a supplemental test.

b) In blood-banking only donated blood is screened by a single ERS as per recommendations of Govt. of India. Therefore, these results should not be used to identify the indiviudals.

c) In serosurveillance studies since the positive results of first ERS are validated by a second ERS and not by a supplemental test, these results may not be used to identify the asymptomatic individuals.

d) For making a diagnosis of HIV-infection, the recommendations of the Govt. of India (chapter 9) must be followed.

e) HIV test results should be reported to the physician who has requested the test. All HIV testing, in which an individual is identified, must be preceeded and followed by pre-test and post-test counselling respectively.

f) All test results must be kept confidential and should never be discussed in public. The test results should never be communicated on telephone.

10.3 PROFICIENCY TESTING OF LABORATORY

Proficiency testing is synonymous with External Quality Assessment (EQA). Recently, National AIDS Control Organisation has formulated a Quality Assessment Programme. Under this programme coded panels of known HIV antibody positive and negative sera are provided to the participating laboratories for HIV proficiency testing on a regular basis. These panels are processed and tested within a specified period so that results from all the participating laboratories may be analysed together. The participating laboratories are requested to handle these coded panels in exactly the same manner as the the routine samples for HIV testing in ELISA. This is because the purpose of such a programme is to identify the problems in HIV testing.

While analyzing the results and preparing a comprehensive report, care is taken not to identify any of the participating laboratory either by name or by location. These reports are then transmitted to the participating laboratories where the laboratory in-charge may assess the adequacy of quality assurance and quality control measures in his own laboratory and compare the performance of his laboratory with other laboratories. In case there is a problem he may investigate, identify and take appropriate timely action to rectify the problem.

Since every laboratory aspires to be a respected testing facility it must initiate a good and effective quality assurance/quality control (QA/QC) programme. Effectiveness of such a programme may be assessed by an external quality assessment. However, a good QA/QC programme cannot be substituted by quality assessment programme, but a good QA/QC programme may substitute for quality assessment.

Many a time false negative and false positive results may be due to technical errors

especially if the tests are performed under less optimal conditions. Therefore, it is necessary to monitor the performance of the laboratory workers periodically by incorporating the samples with known results with the routine samples without the knowledge of the laboratory workers. This is done to ensure the accuracy, reliability and reproducibility of the test results reported by the laboratory.

10.4 RECORD KEEPING

a) A laboratory must maintain a log book for recording of the laboratory specimens. The information contained in the log book should be kept confidential.

b) A work sheet (see annexure) containing the identification numbers of sera to be tested must be prepared each time before the test run is performed.

c) Daily records of temperature of water baths, incubators, refrigerators and freezers should be maintained.

d) Micropipettes should be calibrated atleast annually.

e) ELISA readers should be calibrated to ensure accuracy of their readings.

f) The laboratory should maintain a file where all procedures/package inserts are kept for ready reference.

g) Numbers of ELISA positive samples being reported by each laboratory from various high risk group, moderate risk group and low risk group should be maintained.

h) Number of ELISA positive samples which have been found negative by Western blot assay should be maintained.

i) Number of times an ELISA positive serum sample was rerun before subjecting it to supplemental test should be maintained.

These data will reflect upon the technical problems if any existing in a laboratory.

10.5 LABORATORY ASPECTS

10.5.1 Specimen collection and processing

Five ml blood is collected by venepuncture using either a disposable plastic syringe or an autoclaved glass syringe. After collecton of the sample the disposable syringe is properly discarded, whereas the glass syringe is kept in a beaker containing liquid bleach disinfectant for 10 to 15 min before resterilization. Since lipaemic, haemolysed and contaminated serum samples do not yield reliable results, care should be taken to collect blood, fasting or after a light breakfast, in a dry sterile container. Haemolysis can be avoided by a) not leaving the tourniquet on the arm for more than 1 min; b) not forcefully transferring the blood through a needle to a tube. Universal precautions including wearing gloves must be followed while drawing the blood. It is very important to prevent infection being passed from an individual to the laboraroty worker and vice-versa. Labelling of the sample for proper identification of the individual is extremely important to avoid incorrect reporting of HIV status. Therefore, the tube must be labelled properly immediately upon collection of blood sample. The sample is allowed to sit at 37°C for 30 min and then 4°C for clot retraction.

Thereafter, the sample is spun at 1500 rpm at 4°C and the serum is collected aseptically

in a sterile container. No preservative is added. In case it is not tested for HIV antibodies, it may be stored at 4°C for 1-10 days or at –30°C for long term storage. Repeated freezing and thawing should be avoided.

10.5.2 Preparation of the external controls

Positive and negative controls: One unit of blood from each donor, tested negative for hepatitis B surface antigen, but tested (i) positive for HIV antibody by ELISA and Western blot, or (ii) negative for HIV antibody (out dated blood sample), or (iii) positive for HIV antibody by ELISA but negative by Western blot assay (False positive result) is collected from the blood bank. The plasma is separated, heat inactivated at 56°C for 30 min and then recalcified. It is then incubated at 37°C for clotting to occur. The clot is spun down and the serum is separated aseptically. It is filtered through a millipore filter (0.2 um or 200 nm pore size) to remove any bacterial contamination. No preservative is added. It is aliquoted, labelled properly and stored at –40°C. Once thawed, the aliquot is stored at 2°C to 8°C. It is discarded after using it once. HIV positive serum samples should not be discarded. These should be sent to national reference centres for long term storage in serum bank at –40°C.

10.5.3 Borderline or low positive control

Ten fold serial dilutions (10^{-1} to 10^{-6}) of a known positive serum are prepared in the serum from HIV negative donor which is used as a diluent in place of normal saline or buffered salt solution to keep the proteins in a natural environment. Each dilution is assayed in sextuplicates for HIV antibodies. The mean OD of each dilution and SD are calculated. A graph with OD of each 10 fold dilution is then plotted. The highest dilution of the serum giving positive result in ELISA is taken as an end point. Three dilutions, viz., end point dilution +1 SD, end pont dilution and end point dilution –1 SD are tested by ELISA in sextuplicates and also by WB assay. If these dilutions give reproducible results (intra-run variability), they are tested on six consecutive days for inter-run variability.

Care must be taken not to select a sample with so low antibody titer that the OD fluctuates above and below the cut off due to normal variations. Also the sample with a very high antibody titer should not be selected. It will be of limited use for borderline monitoring.

10.5.4 Recalcification of plasma

Ideal material for quality control and quality assessment is serum. This is because plasma tends to be unstable on long storage and may clot spontaneously during transport. Since under many circumstances blood collected from a blood bank may be the only source for HIV positive and HIV negative samples, plasma must be recalcified to produce serum. However, recalcification should be done with care because excessive recalcification may affect some assays like gelatin particle agglutination, adversely. Only citrated blood should be recalcified.

- Make a 10X Recalcification solution ($CaCl_2.6H_2O$ 55g, $MgCl_2.6H_2O$ 16g, in 100 ml distilled water; autoclave at 121°C for 20 min).
- Add 1.5 ml of recalcification solution to 1 unit of blood or 250 ml of plasma which has been brought to room temperature.

- Incubate at 37°C for 30-60 min (until clot formation).
- Keep at 4°C overnight.
- Spin at 1500 rpm for 20 min.
- Separate the serum aseptically.
- Label and store in small aliquots at –40°C.
- Test small aliquot from each sample for HIV antibodies by ERS tests and Western blot assay.

10.5.5 Preservatives for serum samples

Although Seitz filters may be used to remove bacteria and fungi from the serum samples, filtration of recalcified serum is often difficult as filters tend to clog due to fibrin clots. Centrifugation would remove clots by and large before filtration. Use of prefilters will also help in clarifying the serum samples.

Even though one may start with sterile serum sample, subsequent use and manipulation in the laboratory may easily contaminate it. Therefore, addition of Bronidox L (5-bromo-5-nitro-1,3-dioxane in propylene glycol, Henkel Chemicals) to the serum sample to a final concentration of 0.05% may help in preventing growth of contaminants. Thiomersal (mercuric chloride) is generaly used in a final concentration of 0.01% but it is effective only for a few weeks. This is because it loses its activity especially when exposed to light. Sodium azide should not be used as it inactivates peroxidase conjugate.

10.5.6 Storage of serum

Serum stocks are best stored at –40°C or below in small aliquots of 5-10 ml. Repeated freezing and thawing should be avoided. Once thawed, it should be further aliquoted in smaller size and kept at 4°C until used.

10.5.7 Use of freeze-dried controls

Freeze drying of a serum sample is an effective method for storage of serum sample at 2-8°C for a very long time. In addition, the shipment of freeze dried sample is very simple and straight forward. However, during the process of freeze drying, antibody activity especially of the borderline samples may be lost.

10.5.8 Preparation of proficiency panels

A proficiency panel consisting of two strong positives, two negative and two borderline positives sera samples is prepared from the aliquots of external controls stored at –40°C and coded. If possible, representative serum samples from different areas of the country may be included in the panel.

10.5.9 Tracking the performance of the kit under field conditions

Each laboratory performing ELISA should maintain the following information to ensure

good performance of the kit.

i) OD values of internal and external positive controls of each test run
ii) OD values of internal and external negative controls of each test run
iii) OD values of external borderline positive controls of each test run
iv) Cut off value of each test run
v) Name, batch and lot number of the kit used.
vi) Expiry date of the kit used.
vii) Date of the test run.

This information will help to keep a track of the performance of the kit under the field conditions.

10.5.10 Quality control of HIV antibody test kits

i) Monitoring of different lots of HIV Kit

Sometimes a manufacturer produces a reagent-lot that passes quality control requirement in his unit but fails to perform adequately in the field. This could be due to several factors including artificial stabilization of the controls by the manufacturer, substandard storage and shipping conditions etc. Therefore, it is imperative that these lots of reagents must be identified quickly before distribution to the surveillance centres. This is done by a technique called "Parallel testing" in which performance of new lots of kit with the previous lots via a common control material (external controls and the controls from previous lots) is compared. If all controls produce expected results, the new lot has passed the parallel tests and may be used for routine testing.

ii) Monitoring of different ELISA Kits

There are four main objectives of HIV-antibody screening programme, namely:

a) Safe blood banking
b) Sero surveillance
c) Sero diagnosis
d) Research

For safe blood banking, it is imperative that the ELISA or any other test kit should correctly identify all antibody positive sera. In other words the test kit should have 100% senstivity. The sensitivtiy is generally expressed in terms of percentage which is a qualitative measure. A quantitative expression of sensitivity in terms of positive delta value (delta +) is better and more reliable than percentage value. This helps in selecting among ELISA kits with equal sensitivity. The higher the delta+value, the higher is the probability that this test will correctly identify antibody positive sera.

For sero-diagnosis and sero-surveillance, an ELISA kit with a high sensitivity and high specificity is needed.

A negative delta value (delta-) is quantitative expression of specificity. The greater the negative delta (delta-) value, the higher is the possibility that this assay will correctly identify

the true negative sera.

iii) Calculation of Delta Values

a) To calculate the delta values, 50 confirmed positive and 50 confirmed negative sera are tested.

b) Cut off value is calculated as suggested by the manufacturer.

c) Mean OD and statandard deviation of positive and negative samples is calculated.

d) Mean OD/CO ratio of positive and negative samples is calculate by the following formula

$$\text{OD/CO ratio} = \frac{\text{mean OD of the samples}}{\text{mean CO value}}$$

Delta value is calculated by the following formula:

$$\text{Delta} + = \frac{\text{Mean OD/CO ratio of positive samples (log 10)}}{\text{Standard deviation of positive samples}}$$

$$\text{Delta} - = \frac{\text{Mean OD/CO ratio of negative samples (log 10)}}{\text{Standard deviation of positive samples}}$$

10.6 EVALUATION OF INDIRECT IMMUNOFLUORESENCE ASSAY (IFA) AS A SUPPLEMENTAL TEST

Although the Western Blot (WB) Assay has the advantage of being able to indicate actual antibodies that react with the specific HIV antigens, it is very expensive and time consuming. Sometimes the results are difficult to interpret. In addition, it is very much technique dependent.

On the other hand, the IFA offers several advantages over the WB assay. It is easy to perform, much less expensive than WB and can be completed in a short period of time. The test is easy to read but requires an expertise to do that.

Therefore, IFA may be used as a supplemental test in parallel with WB assay. IFA kits are commercially available.

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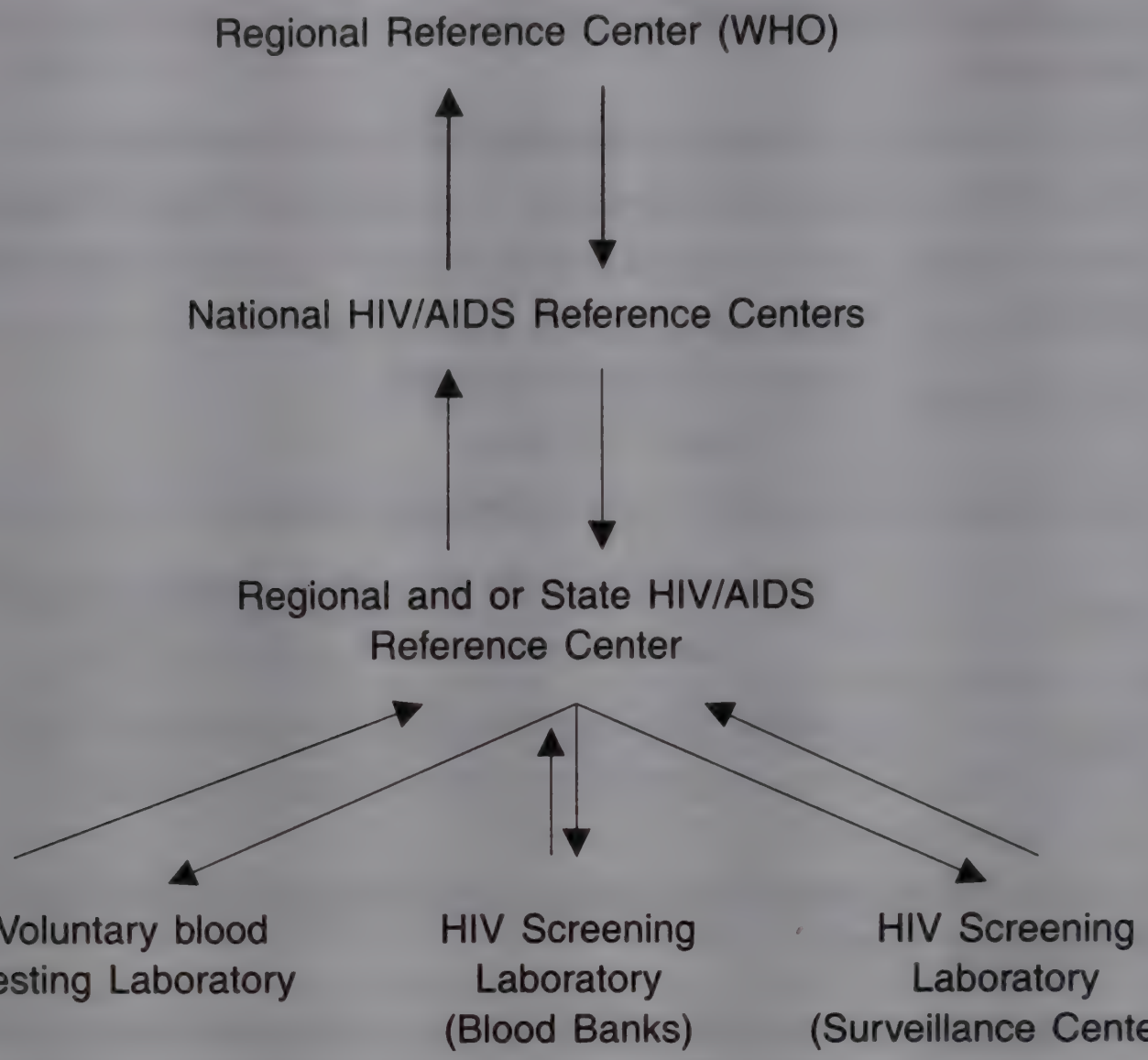
Chapter 10 - Annexure 1

STRUCTURE OF THE QUALITY ASSESSMENT PROGRAMME


Regional Reference Center (WHO)
National HIV/AIDS Reference Centers
Regional and or State HIV/AIDS Reference Center
Voluntary blood Testing Laboratory (Blood Banks)
HIV Screening Laboratory
HIV Screening Laboratory (Surveillance Centers)

Chapter 10 - Annexure 2

QUALITY ASSESSMENT PROGRAMME

Laboratory Enrollment Form

1. Name of the Laboratory

2. Mailing Address

3. Telephone Number Extn.

4. Telex Number

5. Fax Number

6. Laboratory Incharge (Name)

7. Laboratory Incharge (Title)

8. I wish to enrol in the quality assessment programme of the National Reference Centre/ Regional Reference Centre under NACO.

Yes/No

Signature of the Incharge

Please mail the completed form to the concerned National and/or Regional Reference Centre.

Chapter 10 - Annexure 3

Proforma for sending anti-HIV test results by participating laboratory under QAP

ENZYME IMMUNOASSAY RESULTS

Please record the absorbance values (column A) and the appropriate cutoff value (column B) and calculate the sample/cutoff ratios (A÷B) for each panel sample. Also record whether the serum is regarded as positive or negative by the assay.

please circle one: **SCREENING** **SUPPLEMENTAL**

Sample code	Absorbance values (A)	Cutoff value (B)	(A) ÷ (B) 3 decimal places e.g. 2.345,0.567	Test interpretation e.g. negative
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				

Commercial Name of Assay: .....

Name of Manufacturer: .....

Assay Kit Master Lot No.: ..... Expiry Date: .....

Assay Conditions: Temperature (°C) Time (hr: min)

Sample Incubation:

Conjugate Incubation:

Substrate Incubation:

Circle the incubation condition that applies. The microplate was incubated:-

1. In a water bath. 2. In an incubator. 3. On a bench. 4. In a dedicated machine.

Other (give details) .....

Name of Operator: ..... Date: .....

Comments: .....

.....

Chapter 10 - Annexure 4

Proforma for sending anti-HIV test results by participating laboratory under QAP

RAPID ASSAY (PARTICLE AGGLUTINATION/SPOT/DOT BLOT/COMB) RESULTS

Please record if a reaction is observed with unsensitised particles for each serum (see [Control] column). If your laboratory titrates positive sera please record the titre. Note, however that this is not essential, but optional. Format for agglutination assay is given.

please circle one: **SCREENING** **SUPPLEMENTAL**

Sample code	Unsensitised particles (Control)	Sensitised particles (Test)	Titre (Optional)	Positive	Remark
1					
2					
3					
4					
5					
6					
7					
8					
9					
10					

Commercial Name of Assay: .....

Name of Manufacturer: .....

Assay Kit Master Lot No.: ..... Expiry Date: .....

Assay Conditions: Temperature (°C) Time (hr: min)

Sample Incubation:

Circle the incubation condition that applies. The microplate was incubated:-

1. In a water bath. 2. In an incubator. 3. On a bench.

Other (give details) .....

Name of Operator: ..... Date: .....

Comments: .....

.....

Chapter 10 - Annexure 5

Proforma for sending serum panel test results (anti-HIV) by reference centre to the participating laboratory under QAP

Table 1. Details of samples from panel

Panel number	Dilution	Sample type	Sample origin	Anti-HIV-1 status
HIV 20	Undiluted sample	Plasma, lyophilised	NICD (blood donors)	I
HIV 31	Diluted 1:2048	as above	as above	N
HIV 46	Diluted 1:128	as above	as above	I
HIV 89	Diluted 1:16	as above	as above	P
HIV 120	Diluted 1:8	as above	as above	P
HIV 131	Diluted 1:4	as above	as above	P

Table 2. Reference WB/LIA and screening assay test results

Sample number	Western blot and or line immunoassay results										Blot/ LIA	Rapid test	ELISA
	p18	p24	p31	p34	p35	p40	gp41-45	p53	p55	p68			
HIV 20	+	+/–	–	–	–	–	–	–	–	–	I	N	0.70
HIV 31	+/–	–	–	–	–	–	–	–	–	–	N	N	0.62
HIV 46	+/–	+/–	–	–	–	–	–	–	–	–	I	R	0.66
HIV 89	+/–	+/–	–	–	–	–	+	–	–	+/–	P	R	1.28
HIV 120	+/–	+	–	–	–	–	+	–	–	+	P	R	2.09
HIV 131	+	+	–	–	–	–	++	–	–	+	P	R	3.52

Western Blot
Line Immunoassay
HIV dot blot
Capillus (latex agglutination)
Immunocomb

P: positive
N: negative
R: reactive
I: indeterminate

Chapter 11

EVALUATION OF HIV KITS AND PREPARATION OF SERUM PANEL

11.1 INTRODUCTION

Periodic evaluation of HIV test kits is a prerequisite of a good quality management. A large number of assays are available commercially each offering essential as well as attractive performance characteristics namely sensitivity, specificity, efficiency and predictive values. However, the performance of the test will depend upon the conditions in the laboratory, technical expertise, population being tested etc. So, wherever possible, evaluation of kits should be done under the same conditions and in the same locale using the local panel of sera to assess the performance characteristics and efficiency of a test kit. New and improved kits are also being developed continuously and these also need evaluation in the same way as the established kits. Reports of evaluation of kits provide a feed back to the manufacturers to improve their products so that the kits may be fit for use under different situations and in different geographical areas. To have an idea about the performance characteristics of these HIV kits, prior evaluation by some notified agency should be made and is mandatory. Situations under which these kits may have to be evaluated can be as follows:

- Kits under import; evaluation with local serum panel is very important.
- New kits developed/manufactured indigenously; to assess efficacy.
- To resolve controversies regarding discordant results or deterioration of components and reagents. This may be due to break in cold chain during transit and/or storage of in-use kits

As far as the first category is concerned Drugs Controller General of India should ask the importer to get the representative sample of kits evaluated, using a locally made and well characterised panel by the Institutes identified for this purpose. If found suitable, only then the kits should be allowed to be imported for distribution, both in Government as well as private sector.

In second case, when the kits are manufactured indigenously, the performance characteristics of the kits should be ascertained by evaluating different batches of kits (the final product) with the standard serum panel for evaluation of kits at least at two to three different geographical sites and consistent results should be obtained. Only the kits performing to the standards (sensitivity and specificity etc.) as laid down should be licensed for marketing.

In the third case, the licensed, and/or approved kit's performance may be questioned particularly when different centres report inconsistent results. This can happen due to break in cold chain during transport and storage of HIV kits. To resolve this problem the kits have to be evaluated by the identified centres to ensure optimal performance.

This chapter gives the basic guidelines to be followed to ensure proper evaluation of HIV test kits.

11.2 PREPARATION OF PANEL

A serum panel is defined as a collection of well characterised serum samples for a specific reference/purpose.

Serum panel can be prepared and used for different purposes. These are:

- Panel for evaluation of kits i.e. evaluation panel including seroconversion panel
- Panel for proficiency testing (external quality assurance)
- Panel for quality control in the laboratory (internal quality assurance)

These panels are used for kit evaluation, external quality control assessment and for internal quality control in HIV testing laboratories.

11.2.1 Volume of panel samples

The volume of serum being processed to prepare panel should be sufficient to meet the requirements for at least 2-3 years for the purpose for which the panel is prepared. For example 6ml of serum will be sufficient to evaluate approximately 100 kits.

11.2.2 Collection of specimen for panel

Ideally material used for panel should be serum although plasma can also be used. However, plasma tends to be unsuitable as it clots spontaneously on storage. Therefore plasma should be defibrinised with thrombin or recalcified and then processed for making the panel (refer to chapter 10 for recalcification procedure).

11.2.3 Aliquoting of serum

Repeat freezing and thawing of specimens affects the quality of the panel. To avoid this samples should be aliquoted in volumes that may meet the particular panel requirement at one time. Suitable anti-bacterial agents e.g. Bronidox 0.05% (5-Bromo-5-Nitro-1,3 Dioxane, Sigma chemicals) should be added to panel samples to prevent contamination. The solvent for Bronidox is propylene glycol.

11.2.4 Characterisation of serum panel

Serum samples should be well characterised so that there should not be any doubt about the final outcome of the test results. Specimens should be tested using high quality HIV antibody ELISA or any other screening assay followed by confirmatory Western Blot assay to determine their true status and should also distinguish between HIV-1 and HIV-2 types. As characterisation of each unit in the panel is quite expensive, use of large volume of each specimen will be more cost effective.

11.2.5 Storage

Aliquots must be stored at or below –70°C. Glass vials should not be used as they may eventually crack with long term storage in frozen condition.

11.2.6 Transport of panel

To provide safety for laboratory staff, couriers and community, specimens should be transported under International Air Transport Association (IATA) regulation, for this purpose. Specimens should be packed, labeled and documented as classed 6.2 UN 2814 to minimise the risk of infection to anyone during transit.

11.3 COMPOSITION OF SERUM PANEL

These are well characterised serum samples, those which have given consistent results with all the available HIV test kits. These sera should be from individuals in whom the true HIV status (infected or not infected) is known with high degree of certainty. Such samples are called "pedigreed sera." Panel must include reactive, nonreactive, weakly reactive (grey zone) and indeterminate classified sera. Both HIV-1 and HIV-2 reactive sera should be included in the panel. The panel should be representative of the population/area where the kits are going to be used.

To prepare a local panel, sera should be tested with all the available kits.

- At least 50 specimens should be included in each panel of reactive, weakly reactive, indeterminate and non reactive. The panel size can be increased as more the number of sera in each panel, more statistically valid is the comparison.
- Non reactive panel should also include representative sera from individuals suffering from local endemic diseases (e.g. malaria, tuberculosis, hypergammaglobulinaemia etc.).
- Panel specimens should be appropriately processed/characterised and stored as aliquots in frozen condition. All specimens should be in the same condition as far as storage is concerned when test for evaluation of kits is performed.
- Specimens should be nonhaemolysed, nonlipaemic and nonturbid etc.

11.4 REFERENCE TEST

A well established, reference gold standard test must be available to the laboratory for comparison, the results of which should correlate with the actual status of the patient. Currently, such a test in serologic testing for HIV infections is considered to be Western blot (WB) test. However, sometimes WB may give indeterminate results in which case more sophisticated tests like synthetic peptide based Line Immunoassay, polymerase chain reaction (PCR) and virus culture can be undertaken. The tests are expensive, labour intensive and require higher level of technical expertise. Thus for the moment WB usually is regarded as the Referene/Gold standard test. When a number of kits are being evaluated the tie breaker for false positive/false negative is usually a WB test, sometimes supported by more sophisticated tests as mentioned above.

11.5 TESTING CONDITIONS

All evaluations must be performed under identical conditions. The tests must be conducted in the same laboratory using same equipments (pipettes, instruments etc.), by the same technician, using kits which have not expired and have been stored properly and using the exact protocols provided in the package insert.

The technicians must not know the results obtained on the panel with other kits i.e. the evaluation testing is done blindly.

The parameters to be calculated to assess the performance characteristics of the kits include:

- Sensitivity
- Specificity
- Efficiency
- Delta values
- Positive predictive value
- Negative predictive value

11.5.1 Sensitivity

Sensitivity of a test is defined as its ability to detect truly infected individuals as also its ability to detect very small amounts of analyte. Sensitivity can be calculated by the following formula:

$$\text{Sensitivity} = \frac{\text{True positives}}{\text{True positives + False negatives}} \times 100$$

or

$$\text{Sensitivity} = \frac{\text{Number of samples detected as positives by the kit under evaluation}}{\text{Total number of positives by the reference test employed}} \times 100$$

Example:

Total sera tested	=	100	
HIV infected	=	10	↑ Results with the
HIV noninfected	=	90	↓ reference test

Results with kit under evaluation: 9 out 10 HIV infected detected as positive

Sensitivity of the kit under evaluation will equal to $\frac{9}{9+1} \times 100 = 90\%$

11.5.2 Specificity

This is the ability of an assay to correctly identify all the uninfected individuals i.e. there should be no false positives (reactive result in the absence of disease).

The specificity can be calculated by the following formula:

$$\text{Specificity} = \frac{\text{True negatives}}{\text{True negatives + False positives}} \times 100$$

or

$$\text{Specificity} = \frac{\text{Number detected as negatives by the kit under evaluation}}{\text{Total number of negatives detected by reference test employed}} \times 100$$

Example:

Total sera tested	=	100	
HIV infected	=	10	↑ Results obtained
HIV noninfected	=	90	↓ with reference test

The results obtained with kit under evaluation, 9 positive out of 10 HIV infected and 1 positive out of 90 HIV noninfected.

Specificity of the kit under evaluation will equal to $\frac{89}{89+1} \times 100 = 98.9\%$ (approximately)

11.5.3 Efficiency

It is the overall ability of a test to correctly identify all positives as positive and all negatives as negative. Efficiency is determined as below:

$$\text{Efficiency} = \frac{\text{True positives} + \text{True negatives}}{\text{True positives} + \text{False negatives} + \text{True negatives} + \text{False positive}} \times 100$$

or

$$\text{Efficiency} = \frac{\text{Total number detected as positives + as negatives by the kit under evaluation}}{\text{Total number of positives + negatives detected by reference test employed}} \times 100$$

Example:

Total sera tested	=	100	
HIV infected	=	10	↑ Results obtained
HIV noninfected	=	90	↓ with reference test

The kit being evaluated detected 9 out of 10 HIV infected as positive and one out 90 noninfected also as positive.

So efficiency of the kit will equal to $\frac{9 + 89}{9 + 1 + 89 + 1} \times 100 = 98\%$

11.5.4 Delta value

This is a statistical measure of sensitivity and specificity and is calculated from the actual OD values generated by the test. This also helps to determine how close the O.D. value of false positive and or false negative is to the real positive and or real negative value, respectively. If these values are too close the test is flawed.

Delta values are defined as the distance of the mean O.D. ratio of the sample population from the cut off (C.O.) measured in standard deviation units.

$$\text{Delta values} = \frac{\text{Mean O.D./Cut off ratio (log 10)}}{\text{Standard deviation}} \times 100$$

Positive delta value is calculated on mean of ODs of positive samples and negative delta value is calculated on mean ODs of negative serum samples. When two tests are being compared, the test that has a higher positive delta value would characterise positive samples more accurately; similarly the test with higher negative delta value will better classify the negative samples.

Example:

	Positive samples	Negative samples
O.D./C.O.	4.20	.03
	4.60	.02
	5.40	.04
Mean O.D./C.O.	4.73	.03
Standard deviation	0.61	.01
Log10 of the mean O.D./C.O.	0.67	–1.52
Delta	Positives delta value	Negative delta value
values	+1.10	–152

11.5.5 Predictive values

This is the measure of value of a test in relation to the prevalence of the disease in the population. The value of a test in addition to the parameters described above depends on the population being tested.

Positive predictive value (PPV) is the ability of the test to identify actually infected individuals among all persons giving a positive result with the kit being evaluated.

$$\text{PPV} = \frac{\text{True positives}}{\text{True positives + False positives}} \times 100$$

Negative predictive value (NPV) is the ability of the test to correctly identify the really non infected individuals from among all the persons giving negative result.

$$\text{NPV} = \frac{\text{True negatives}}{\text{True negatives + False negatives}} \times 100$$

If the prevalence is high the PPV of a test will be high i.e. an individual testing positive will mean real infection. Whereas, in low prevalence area the chance that an individual testing positive is really infected is lower.

Example:

Low prevalence area 1%

Total sera tested = 1000

HIV infected = 10 ↕ with the reference test

HIV noninfected = 990

Results obtained with kit under evaluation: Positive results obtained in 8 individuals, two from HIV infected group and 6 false positives from HIV noninfected group.

Negative results obtained in 992 individuals, 984 from the noninfected group and 6 false negatives from HIV infected group.

$$\text{The PPV} = \frac{2}{8} \times 100 = 25\%$$

$$\text{The NPV} = \frac{984}{992} \times 100 = 99.3\%$$

Example:

High Prevalence area 10%

Total sera tested = 1000

HIV infected = 100 ↑ with the

HIV noninfected = 900 ↓ reference test

Results obtained with HIV kit under evaluation; 105 tested positive, 95 from HIV infected group and 10 from HIV non infected group i.e. gave false positive result.

Negative result was obtained in a total of 900 cases. 892 of 900 were from HIV noninfected group and 8 were from HIV infected group.

$$\text{PPV} = \frac{95}{105} \times 100 = 90.5\% \text{ (approx)}$$

$$\text{NPV} = \frac{892}{900} \times 100 = 99.12\%$$

All the above parameters are ascertained by the laboratory carrying out the evaluation of kits. The parameter's values should be within those recommended by the technical expert committee, G.O.I. on this subject. The final approval of the test kit is given by DCGI on the basis of the evaluation reports submitted by the identified evaluation centres.

Chapter 12

LABORATORY TESTS FOR MONITORING STAGE AND PROGRESSION OF HIV INFECTION

12.1 INTRODUCTION

Infection with the human immunodeficiency virus (HIV) may develop to acquired immune deficiency syndrome (AIDS) at different rates in different individuals, with a spectrum varying from rapid progression to long term non-progression. This variability makes it essential to have tests which can accurately assess the stage of infection in an individual, as well as predict its course and monitor its progression. These laboratory tests are very valuable during the period of clinical latency, and, subsequently, supplement various clinical parameters, which are also extremely important in categorizing the infection/disease stage. Response to anti-retroviral therapy is also monitored using these prognostic tests.

12.2 LABORATORY TESTS

The laboratory tests used for monitoring stage and progression of HIV infection can be classified into:

Viral markers

- Plasma HIV RNA load
- Viral antigenaemia e.g. p24 antigen

Surrogate markers

- Virus specific markers e.g. antibodies to HIV antigens (e.g. p24, p17, nef, etc.)

Non-specific markers

- Cellular markers e.g. CD4 cell count, phenotypic markers of lymphocyte activation.
- Soluble markers of immune activation e.g. neopterin, beta 2 microglobulin and some cytokines and their receptors in serum.

The basis of many of these laboratory tests is that HIV induces immune system activation, beginning in the early stages, which progresses throughout the course of HIV infection. This can be detected by changes both in serum levels of soluble immune activation products and in several phenotypic markers of lymphocyte activation. Although such immune activation is usually beneficial to the host in other infections, it can be deleterious in infection with HIV. Lymphocyte activation triggers HIV replication, releases virus from cellular reservoirs and contributes to disease pathogenesis. The increased rate of HIV replication is reflected in an increase in plasma viral RNA load, which is presently considered the most representative and sensitive laboratory test for monitoring progression of HIV infection.

Increased HIV replication leads to a depletion of CD4 cells, which are its main target. CD4 cell count, a very useful surrogate marker, was one of the earliest to be described and is still used for staging infection and monitoring its progression.

12.3 VIRAL MARKERS

12.3.1 Plasma HIV RNA load

Plasma viral load (HIV RNA) quantification is presently considered the best method for monitoring progression and response to anti-retroviral therapy. Active replication of virus occurs in all clinical stages of infection. It is possible to detect and quantify virus throughout the course of HIV infection. The viral load usually ranges between 10^2 and 10^7 HIV RNA copies/mL in untreated individuals though it may be lower in those on treatment.

The techniques available for quantifying viral RNA are:

- Quantitative RNA-PCR
- Branched DNA assay
- Nucleic acid sequence based amplification

Methods and principles

12.3.1.1 Quantitative RNA PCR (Amplicor HIV-1 Monitor Test, Roche)

This test is based on parallel amplification of HIV RNA (142 bp sequence from gag gene) from the sample and a known amount of quantitation standard (QS) RNA, using RT-PCR (reverse transcriptase-polymerase chain reaction) with the same set of biotin-labeled primers to give different products (amplicons). These are detected in separate wells of a microwell plate by hybridization with immobilized HIV and QS specific probes. The bound biotinylated amplicon is quantified colorimetrically by measuring the optical density (OD), obtained by using avidin-horse radish peroxidase (HRP) conjugate and an appropriate substrate. The HIV RNA copy number can be then calculated by extrapolating from the known input number of copies of QS RNA. To cover a whole range of HIV RNA copies possible in plasma of infected persons, a number of dilutions are put up (**Fig. 12.1**).

12.3.1.2 Branched DNA assay (Quantiplex b-DNA, Chiron)

The branched DNA assay is based on amplification of the signal instead of the target. A target probe complementary to both the target sequence and the capture probe (immobilized on the solid phase) hybridizes with both of these. Another sequence on the target probe (now attached to the solid phase through the target) then hybridizes with an "amplifier" branched DNA (directly or via an intermediate extender probe). An enzyme-labeled probe, which can bind to the multiple branches of the amplifier branched DNA, is allowed to hybridize with it. This amplified label is detected by a luminometer and quantified by its action on a chemiluminiscent substrate. The quantity of the target in the sample can be determined from this (**Fig. 12.2**).

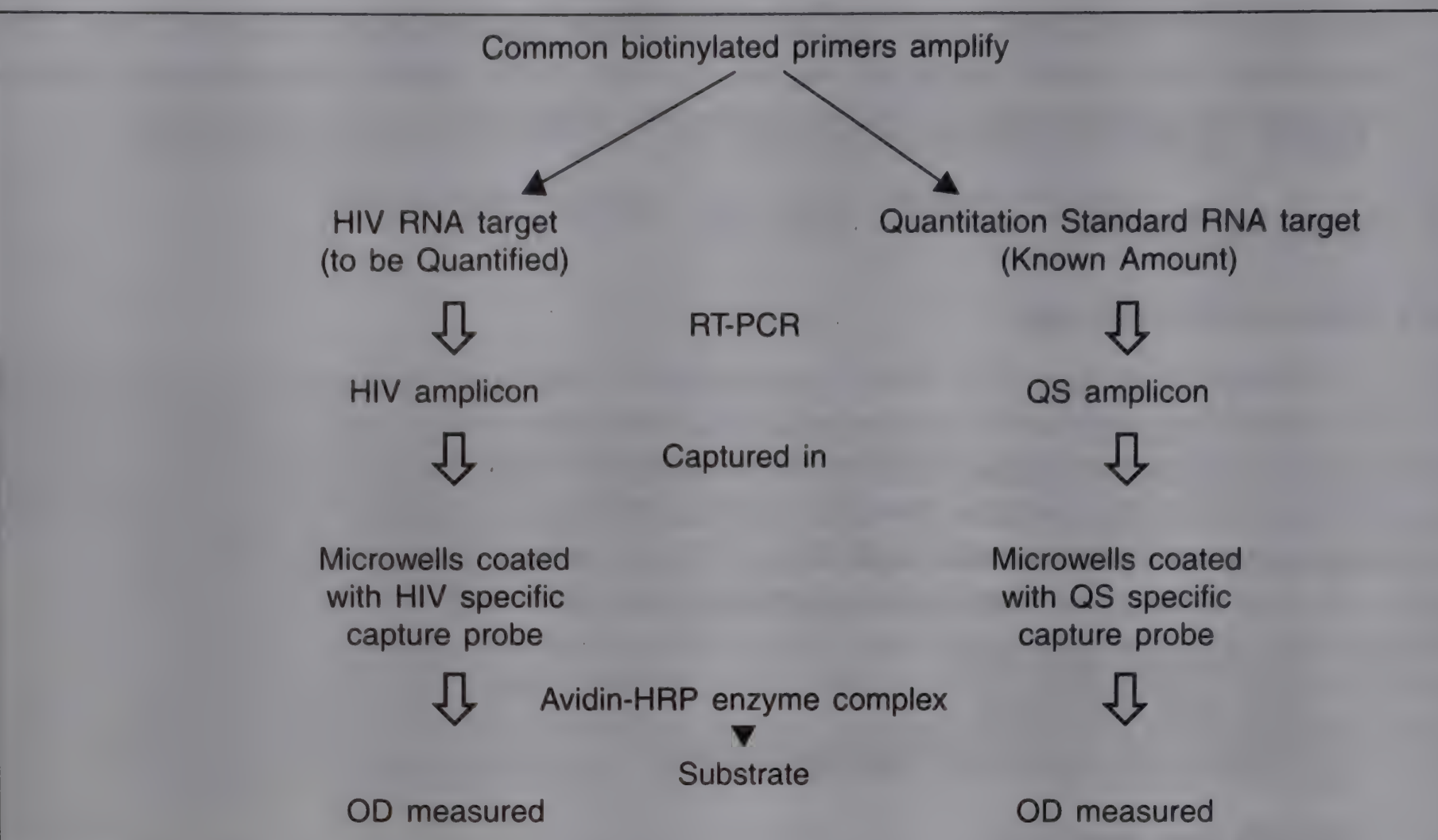


Fig. 12.1 Quantitative HIV RNA PCR for viral load measurement

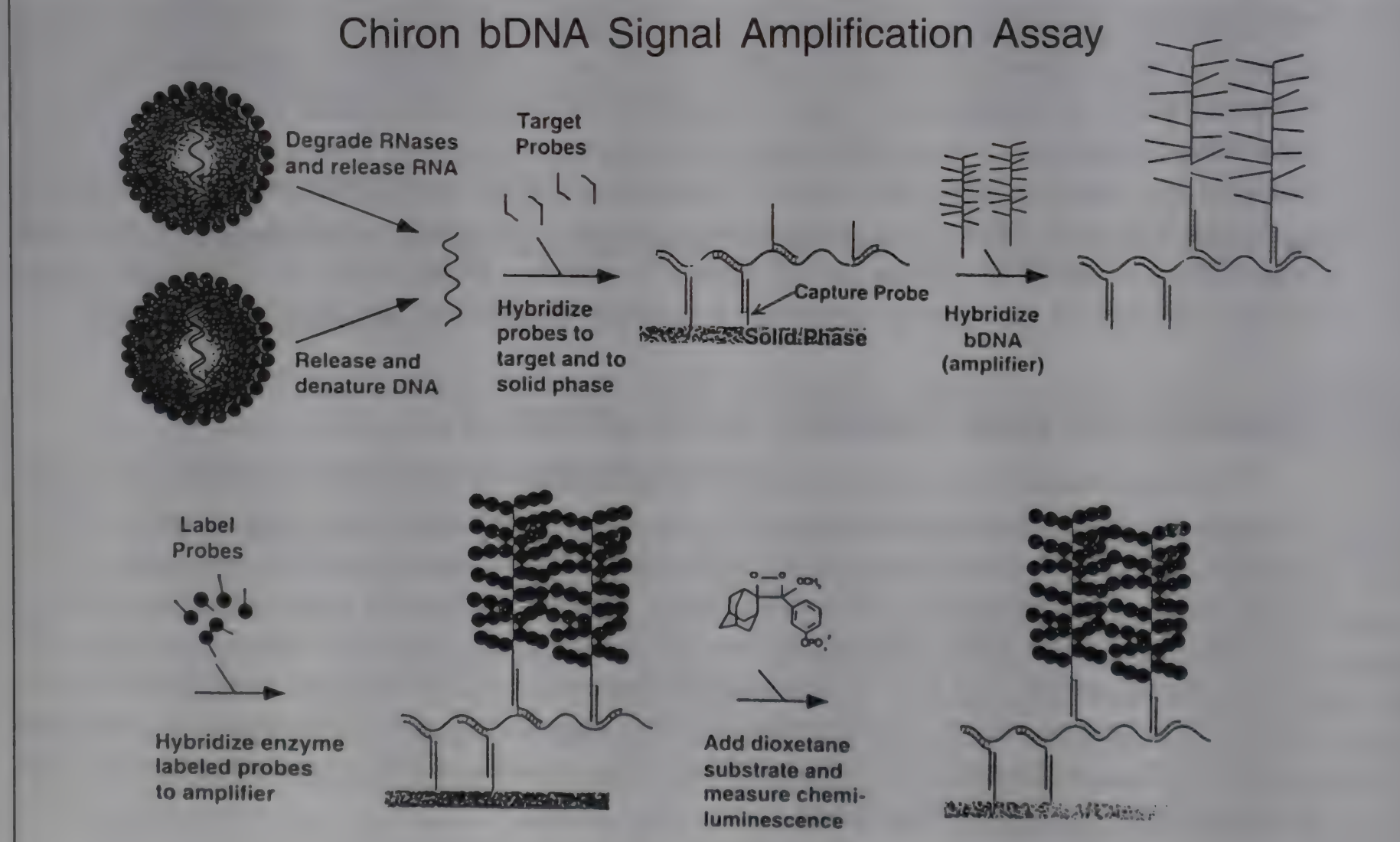


Fig. 12.2 Branched DNA assay

12.3.1.3 Nucleic acid sequence based amplification (NASBA, Organon Teknika)

This technique uses 3 enzymes - reverse transcriptase (AMV-RT), RNAse H and T7 RNA polymerase - at a single temperature (37°C), to generate multiple copies of the target HIV RNA, by a strategy similar to that used by retroviruses for their replication. RT enzyme and a primer (complementary to HIV RNA target) designed with the T7 RNA polymerase promoter sequence at one end, are utilized for synthesis of an RNA-cDNA hybrid containing the T7 promoter from the target. From this, the original RNA target strand is removed by addition of RNAse H and dsDNA is generated by the DNA polymerase activity of RT and a second primer. Multiple copies of RNA are then generated by T7 RNA polymerase enzyme. This RNA enters another similar cycle and the amount of RNA multiplies exponentially with each cycle (**Fig. 12.3**). With appropriate controls and standards it is possible to quantitate the original RNA target by this method.

Present status of HIV RNA load

Plasma HIV RNA levels are more than 10-fold lower in long-term non-progressors (mean value: 70,000 copies/mL) than in individuals with progressive disease (mean value: 1,000,000 copies/mL). A viral load of >100,000 copy equivalents/mL by b-DNA assay, within 6 months of seroconversion increases the risk of progression to AIDS within 5 years by more than 10-fold. Persistently detectable viraemia and high baseline levels carry a poor prognosis, while risk of progression is low at copy numbers < 10,000/mL. Apart from being

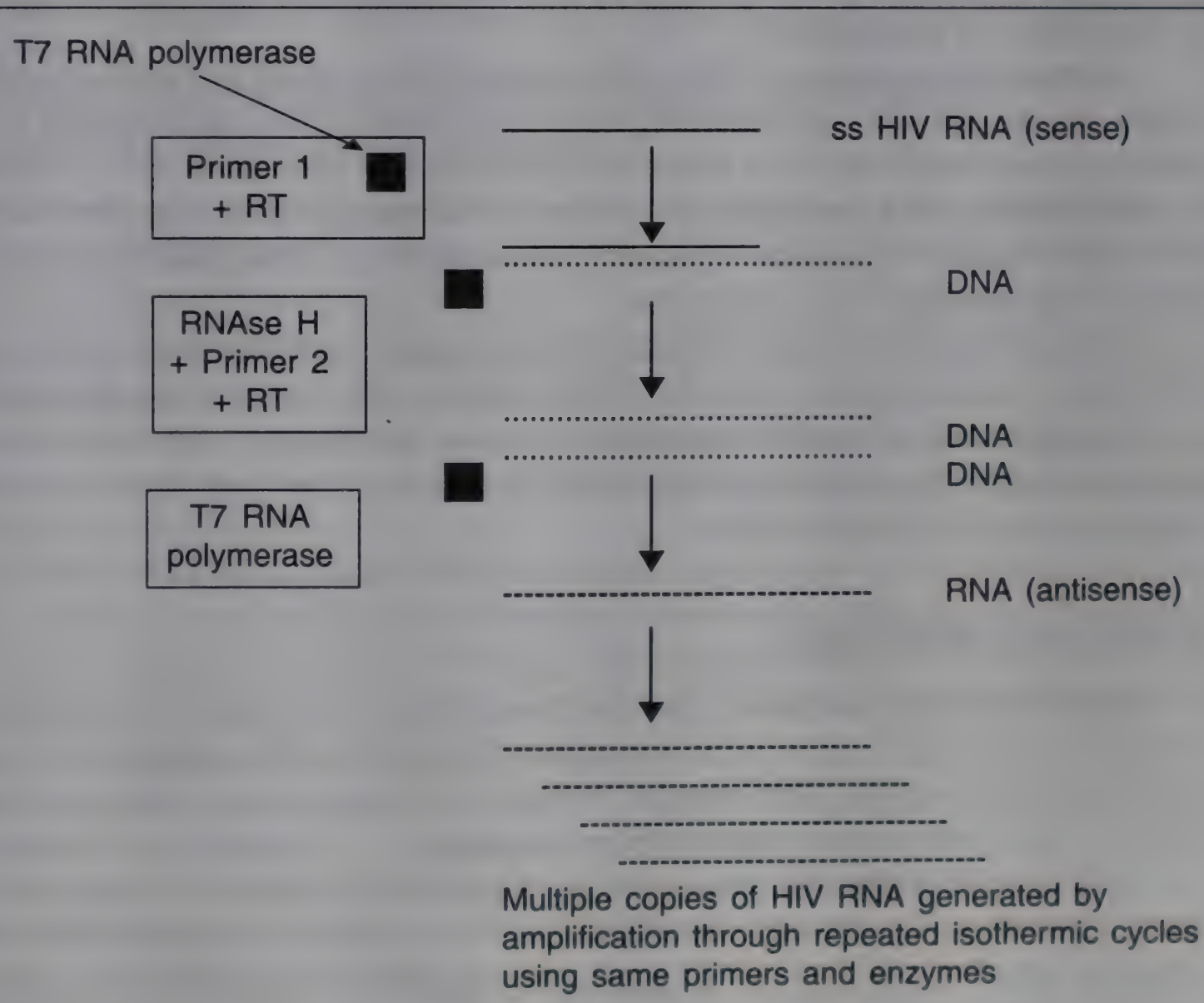


Fig. 12.3 NASBA

an extremely useful predictor of progression, viral load responds very well to antiretroviral therapy and can be used to monitor it.

The variation between different methods for HIV RNA load quantitation was between 0.08 and 0.20 $\log_{10}$ by comparative evaluation in a multi-centric study, with a greater variation with clinical samples than with spiked samples. The use of a common set of HIV-1 RNA standards eliminates differences in absolute HIV-1 RNA copy number.

The b-DNA assay had less problems with detection of different HIV-1 subtypes as it assesses a larger portion of the HIV sequence using multiple probes. The quantitative RT-PCR kits give more accurate results in the hands of technicians experienced in PCR, as all the precautions normally taken for any PCR based test are essential to follow.

It is recommended that blood samples should be collected in EDTA (rather than ACD or heparin) and that plasma is better than serum (and usually shows higher values). Plasma should preferably be separated on the same day and stored at –70°C (plasma is stable at 4°C for up to 5 days and at –20°C for up to 20 weeks). The value of viral RNA load (as also most other markers) in monitoring paediatric patients needs further validation.

12.3.2 p24 antigenaemia

HIV gag gene encoded core protein antigen, p24, is one of the earliest viral antigens detectable in the blood after infection. It is useful for diagnosis during the "window period" during early infection and in the newborn. Levels of free antigen decline after the appearance of anti-p24 antibodies and formation of immune complexes. An increase in free p24 levels is a predictor of progression.

Method and principle: ELISA p24 antigen capture assay kits are available for detection of p24 antigen. To increase sensitivity, most kits presently used for diagnosis include immune complex dissociation (ICD) at acidic pH (pretreatment with dilute HCL or glycine, followed by neutralization) as a part of the protocol of p24 detection, to increase sensitivity. Quantitation for monitoring p24 is possible by testing serial dilutions of the plasma/serum. Sensitivity of detection is between 10 to 50 pg/mL.

Present status of tests for p24 antigenaemia: Until the availability of tests for HIV RNA load, it was the main viral load assay available. The antigen is poorly quantifiable, may not be detectable in many individuals and does not change very well with anti-retroviral therapy. It has a limited role in diagnosis (in specific situations) and is not considered as useful a prognostic marker now.

12.4 SURROGATE MARKERS

Virus specific markers: A viral characteristic associated with progression is the conversion of strains from a non-syncytium inducing (NSI) phenotype to a syncytium-inducing (SI) phenotype. This change of character has now been associated with the ability to infect cells with certain chemokine coreceptors.

HIV specific antibodies have also been used as markers of progression, though they are not considered particularly sensitive. Decline in or absence of antibodies to various HIV antigens including p24, p17, gp120, gp41 and nef gene product have been used as surrogate markers in the past.

Non HIV-specific markers: Infection with HIV-1 has a dramatic effect on the immune

system: the loss of CD4 cells and the activation of the immune system, both of these effects are thought to play a role in the development of clinical AIDS. Immune stimulation is reflected by hypergammaglobulinaemia, expansion of macrophage and increase in (activated) CD8 cells. It also leads to a change in the levels of soluble markers of immune activation and some cytokines and their receptors. CD4 cell numbers are decreased in HIV infection but CD3 (total T cell) number remain constant.

A number of non HIV-specific cellular markers have been used for staging, monitoring progression of HIV infection and assessing response to therapy. One of the most useful and commonly used cellular markers is the absolute count (or percentage) of CD4+ lymphocytes in the blood. Other lymphocyte phenotypic markers associated with progression include an increase in indicators of immune activation on T lymphocytes like CD38 (especially on CD8+ lymphocytes), HLA-DR, IL-2R, CD45RO and markers of apoptosis (e.g. Fas). A flow cytometer with at least ≥ 2 colour analysis facility is a necessary requirement for these markers.

12.4.1 CD4 count

The most commonly used cellular marker is the CD4 lymphocyte count. Its decline is the hallmark of HIV infection and the rate of loss in each person is unique. CD4 cell number changes during HIV infection in the following stages:

- Rapid decline for 6-18 months at the time of seroconversion
- Plateau of gradual decline that can last at least several years during the asymptomatic period
- Steeper decline for several months just before AIDS develops
- Continued CD4 cell decline until death.

CD4 cell count is extremely important in the staging of HIV infection and a revised classification of the Center for Disease Control, Atlanta, USA (1993) divides HIV positive persons into three CD4 count categories: 1 = ≥ 500/μL; 2 = 200-499/μL; 3 = < 200/μL (along with 3 parallel clinical stages – A, B and C). A low CD4 count (less than 10%), a number less than 100/μL and a low CD4/CD8 ratio (< 0.2) are highly predictive for death from AIDS-related complications. It can supplement viral load as a marker of progression. A persistently high viral load is predictive of a poor prognosis, especially when accompanied by a very low CD4 count.

However, the clinical benefit of antiretroviral drugs is not well explained by CD4 counts (unlike viral RNA load) and it is less effective in monitoring therapy. It is even less likely to be of use in evaluation of immunotherapy. Sustained increases in CD4 counts with newer multidrug regimens are more effective in predicting a positive response to therapy than the transient increases achieved by earlier regimens.

Methods and principles:

12.4.1.1 Flow cytometry

Signals are generated by cells or particles in suspension passing through a light (usually laser) source in the flow cell, in a single file (aligned by the sheath fluidic system), and are analyzed electronically in a flow cytometer. The parameters measurable include

forward scatter (an indicator of cell size), side scatter (an indicator of granularity of the cell) and signals from multiple fluorescent dyes (e.g. FITC, phycoerythrin) tagged to cell surface phenotypic marker-specific antibodies.

The whole blood lysis method is preferred for analysis of lymphocyte populations. Blood is ideally collected in EDTA and processed on the same day (also for hematological tests).

- A volume of buffer (e.g. about 50 µl of PBS, at room temperature) is dispensed into tubes.
- This is followed by the addition of recommended volumes (usually 10 µl for most commercial preparations) of each fluorescent antibody (depending on the combination to be used) and 50 to 100 µl of whole fresh blood.
- After gentle vortexing, the tubes are incubated at room temperature for 10-20 minutes.
- This is followed by lysis of RBCs and fixation of cells (single-step or as separate steps with washing and centrifugation in between, depending on the reagents used). If commercial reagents are not used then ammonium chloride solution can be used for lysis and paraformaldehyde as fixative.

After fixation the sample should be analyzed immediately if possible, though it remains stable for 24 hours. The calibration control beads (commercially available) are run in the flow cytometer to check the settings, prior to analyzing the sample.

The ideal recommended panel of antibodies for lymphocyte phenotyping by two-colour analysis (using combinations of 2 monoclonal antibodies to different markers and with different labels, per tube) is as follows:

- CD45 (pan leukocyte marker)/CD14 (monocyte marker)
- CD3 (pan T cell)/CD4
- CD3/CD8
- CD3/CD16 ± CD56 (NK cell markers)
- CD19 (B cell marker)
- Isotype controls (IgG1/IgG2)

The gate for analysis may be set on the lymphocyte population by FS vs. SS (lymphocytes separate out to the left of and below granulocytes as they are smaller in size and less granular, but monocytes lie much closer to them). CD45/14 staining helps to further define the gate in which the cells are to be measured and ensures lymphocyte purity, excluding monocytes (at least 85% of cells in the FS/SS gate should be lymphocytes i.e. $CD45^{bright}$,$CD14^{-}$). This and the CD3/CD4 combination is essential for CD4 counting. The CD8 cells, B cells and NK cells may be counted to confirm if the totals "add up", for further accuracy. Isotype controls may not be necessary if the positive cell population is stained brightly and is distinct from the negative population. Otherwise these will be required to set the level where autofluorescence and non-specific fluorescence ends and specific fluorescence begins.

12.4.1.2 Microsphere assay

A commercial microsphere assay for CD4 count is available as the Manual CD4 Count Kit (Coulter, USA). The steps are as follows:

- Whole EDTA blood (100 μL) is added to a tube.
- This is followed by addition of 10 μL small (0.6 μM) latex microspheres coated with anti-CD14 antibodies, to block monocytes by binding to these.
- Subsequently, 10 μL large (2 μM) anti-CD4 coated beads are added. The mixing and binding is for 2 minutes each.
- After attachment of the larger beads to CD4 cells, 10 μL of the mixture is transferred to another tube with 100 μL of a lysis and staining solution, and mixed for 10-15 seconds.
- This is used to charge the two chambers of a haemocytometer, which is examined microscopically. Only cells with 3 or more large beads attached (rosettes) are counted, and not the cells with small or both small and large beads (which include monocytes).
- The total number of such cells in both chambers is multiplied by 7.3 (a factor accounting for all dilutions and conversions) to give the absolute CD4 count per μL.

12.4.1.3 Enzyme immunoassay (EIA)

Capcellia CD4/CD8 test (Sanofi Pasteur, France) is a one-step immunoenzymatic assay based on capture of T cells by CD2 antibodies bound to the wells of a microtitre plate, instead of solubilization, followed by detection with anti-CD4 or CD8 peroxidase conjugate in an EIA format. The CD4 and CD8 number is obtained by conversion from pmol/L values on a standard curve.

TRAx CD4 test kit (T Cell Diagnostics, USA) is a test based on solubilization of CD4 cells from whole blood by a lysis solution to release its antigen followed by its detection in an ELISA format. The number of CD4 molecules per cell is about 50,000 and it does not vary much. Also, the contribution of CD4 molecules from monocytes is too small (the density of molecules being a tenth of that seen in CD4 lymphocytes) and lies below the sensitivity limit of the test. Therefore, measurement of the number of solubilized CD4 molecules by ELISA can be used to calculate the number of CD4 cells.

Zymmune CD4/CD8 Cell Monitoring Test kit (Intracel, USA) uses non-flow cytometry methods to provide absolute cell counts for both CD4 positive and CD8 positive T lymphocytes in less than 35 minutes. Its proprietary technology uses a mixture of antibody-coated magnetic and fluorescent beads. The magnetic beads isolate the cells of choice and the fluorescent beads provide the signal to count the cells.

Present status of methods for CD4 cell count: Flow cytometry is the most accurate method for determining CD4 cell counts, though it is technically demanding and comparatively expensive both in terms of equipment and reagents. The use of appropriate controls in each run and both internal and external quality control (e.g. through the external quality assurance programme of the International Union of Immunological Societies, known as QASI) are essential.

In single platform technology (e.g.FACScount from Becton Dickinson, Immuno VCS from Coulter), total leukocyte and differential lymphocyte counts are possible on the same instrument as the one which determines the CD4 percentage. These methods are less prone to errors in absolute CD4 counting. Such errors may occur in other flow cytometers which measure the percentage of CD4 cells, and require separate automated or manual measurement of haematological parameters (i.e. TLC and DLC) to generate absolute counts from CD4 percentage. FACScount is, however, capable of only limited analysis (e.g. only

CD3, 4 and 8). These systems are not extendable to studies on other phenotypic markers and are also "closed" in terms of reagent usage. Their main advantage is comparative ease of analysis and accuracy without the need for highly specialized technical skills or a separate haematological counter.

EIA-based and manual methods are easier to perform and less expensive than flow cytometry, but are less accurate. An evaluation comparing the Coulter manual kit with flow cytometry showed a coefficient of correlation of 0.91. These kits have a role in developing countries due to lower cost and simplicity. Another advantage is that single samples can be tested.

12.4.2 Soluble Markers

A large number of soluble markers of immune activation have been evaluated as prognostic indicators in HIV infection. These include serum/plasma levels of neopterin, beta 2-microglobulin, tumour necrosis factor alpha (TNFα), soluble CD8, soluble cytokine receptors (sIL-2R for IL-2, sTNFα RII for TNFα) etc. Soluble receptors are usually more stable than their corresponding cytokines.

12.4.2.1 Neopterin

Neopterin (6-D-erythro-trihydroxypropylpterin) is a product of guanosine triphosphate catabolism which is derived from macrophages and B cells stimulated by interferon gamma and reflects its activity, while being stabler. Neopterin is present in both urine and serum. Urine and serum neopterin levels are elevated in patients with asymptomatic HIV infection as compared to seronegative individuals. It is an early marker of HIV infection. The levels rise further on progression from pre-AIDS to clinical AIDS. Since neopterin levels are stimulated by HIV infection, measurement of neopterin level can be useful in monitoring progression and evaluating antiviral therapy.

However, since it is a non-specific marker, besides HIV infection, high neopterin levels in urine and serum may be found in numerous infections and inflammatory disorders, collagen vascular diseases and in advanced stages of certain malignancies.

Method and principle: The earlier tests available for neopterin assay in serum were based on radioimmunoassay but now convenient kits based on ELISA are available e.g. ELItest Neopterin (BRAHMS Diagnostics, Germany) and from IBL, Hamburg, Germany. The kits are based on competitive ELISA. The plates are coated with polyclonal anti-neopterin antibody raised in animals e.g. sheep/rabbit. The neopterin in the test sample competes with enzyme (alkaline phosphatase or peroxidase)-conjugated neopterin. The enzyme conjugate is detected by addition of the substrate and a lower OD corresponds to a higher level of neopterin in the sample. Neopterin standards with different levels of neopterin are run along with samples to generate a standard curve for converting OD readings to levels in nmol/L.

12.4.2.2 Beta-2 microglobulin

Beta-2 microglobulin (β2-m) is a low molecular weight (11,800 kD) polypeptide that forms the light chain of class 1 major histocompatibility complex antigen present on the

surface of most nucleated cells. It is a nonspecific but relatively sensitive marker for immune activation. The results from several longitudinal studies of high-risk groups have also demonstrated that beta-2 microglobulin is an early marker of HIV infection. Serum concentration above background levels are detected in most infected individuals within 6 months of seroconversion. High serum beta-2 microglobulin levels, especially during the first year after seroconversion to HIV positivity, were associated with progression to AIDS. Its measurement may be useful in evaluating anti-viral therapy.

High levels of beta-2 microglobulin have also been reported in tuberculosis, cytomegalovirus infection and lymphomas, which may be present in the late stage of HIV infection making it important to interpret the data on serum beta-2 microglobulin levels in the clinical context. Elevated levels are also seen in certain HIV high risk groups such as haemophiliacs and drug abusers.

Method and principle: Beta-2 microglobulin is present in most biologic fluids at low concentrations and can be measured in serum or plasma by using commercially available quantitative competitive ELISA based tests. The commercially available kits include Quantikine β2-m (R&D Systems,USA)and β2-m (ORGenTec Diagnostika, Germany).

Present status of soluble markers of immune activation: Tests for soluble markers of immune activation, including these two markers, as well as others like soluble receptors for TNF and IL-2, are available as commercial ELISAs and are comparitively easy to perform and less expensive.

Recent studies have shown that sTNF-RII level was prognostically similar to neopterin and CD4 levels and better than sIL-2R level. At CD4 counts < 200/μL, CD4 and individual soluble markers were found prognostically superior to even HIV RNA load, though viral load was a better marker at CD4 > 200/μL. A combination of CD4 with sTNF-RII or neopterin was also slightly superior to viral load across the range of CD4 values. Furthermore, these soluble markers increase significantly > 6 months before decline in CD4 level and increase in HIV RNA level. This implies that immune activation precedes the increase in viral load and is followed by a rapid and accelerated loss of CD4 cells.

12.5 COST FACTOR AND AVAILABILITY

NOTE: Most of the tests available for prognostic follow-up are proprietary items. The list of manufacturers and authorized dealers in India is not exhaustive and represents information available at the time of writing. It does not imply that the specified items are in any way recommended by the panel of experts.

Viral markers: Roche Amplicor HIV Monitor kit (only reagents) for 24 tests costs about Rs.70,000. It requires a thermocycler and is read colorimetrically.

Address:

Wheecon Instruments Inc.
95, III St., Karpagam Avenue,
R.A.Puram, Chennai-600028.
Fax 044 4940788

NASBA (Organon Teknika) is yet to become available. Other products of this company are available through Infar (India) Ltd.

Address:

Infar (India) Ltd.
12D, Judges Court Road, Calcutta-700027.
Fax 033 4706177

Surrogate markers: A flow cytometer can cost between Rs.25,00,000-Rs.50,00,000 depending on accessories needed. Each antibody or combination for 2-colour flow cytometry costs about Rs.10,000-12,000 for 50 tests. After adding the costs of all reagents including lysis solution, minimum of 2 combinations of antibodies, isotype controls and buffers etc., the reagent cost works out to about Rs.700-800 per sample.

The Manual CD4 Count kit (Coulter) costs about Rs.500 per test.

The EIA based Capcellia CD4/CD8 whole blood kit (Sanofi Pasteur) costs about Rs.25,000 for 43 tests.

Reagents for flow cytometry are available from Becton Dickinson (now also includes Pharmingen) and Beckman Coulter (includes some Immunotech flow products also).

Addresses:

Coulter Products Division
c/o Amersham Pharmacia Biotech Asia Pacific Ltd.
41, Community Centre, Kailash Colony Extension, Zamrudpur, New Delhi-110048.
Fax 011 6212114

Coulter Products Division
c/o Amersham Pharmacia Biotech Asia Pacific Ltd.
18, 2nd floor, Cunningham Road, Bangalore-560052.
Fax 080 2204803

Becton Dickinson India Pvt. Ltd.
Manor Hotel, 77, Friends Colony, New Delhi-110065.
Fax 011 6831783/6824056

The cost of an ELISA kit for 96 tests for the soluble markers, including cytokines and their receptors, is about Rs.20,000-22,000. Kits are available from a number of manufacturers, some of which are listed below.

Addresses:

For R&D Systems products -
Spectral Diagnostics Pvt. Ltd.
C-32, Kirti Nagar, New Delhi-110015.
Tel 011 5458049

For Genzyme products -
Biotech India
B-29/10, Nandigram, Lanka, Varanasi-221005.
Fax 542 313474

For Immunotech products -
OSB Agencies Pvt. Ltd.
2/37-A, Ansari Road, Daryaganj, New Delhi-110002.
Fax 2216736

12.6 SUGGESTED READING

i) CDC. 1997 Revised guidelines for performing CD4+ T-cell determinations in persons with human immunodeficiency virus (HIV). MMWR 1997; 46 (No. RR-2): 1-29.

ii) CDC. 1993 Revised classification system for HIV infection and expanded surveillance case definition for AIDS among adolescents and adults. MMWR 1992; 41 (No. RR-17): 1-35.

iii) Fahey JL, Taylor JMG, Detels R, et al. The prognostic value of cellular and serological markers in infection with human immunodeficiency virus type 1. *N Engl J Med* 1990; 322: 162-72.

iv) Graham NMH. The role of immunological and viral markers in predicting clinical outcome in HIV infection. AIDS 1996; 10 (suppl 5): S21-S25.

v) Salazar-Gonzalez JF, Martinez-Maza O, Nishanian P, et al. Increased immune activation precedes the inflection point of CD4 T cells and the increased serum virus load in human immunodeficiency virus infection. *J Infect Dis* 1998; 178: 423-30.

vi) Nicholson JKA, Velleca WM, Jubert S, et al. Evaluation of alternative CD4 technologies for the enumeration of CD4 lymphocytes. *J Immunol Methods* 1994; 177: 43-54.

Chapter 13

TRANSFUSION TRANSMITTED INFECTIONS OTHER THAN HIV INFECTION

13.1 INTRODUCTION

Blood transfusion is usually life saving but can transmit diseases if blood is not tested properly for the presence of micro-organisms before transfusion. The main diseases transmitted through blood are hepatitis, human immunodeficiency viruses (HIV), syphilis and malaria **Table 13.1** gives the list of main diseases transmitted through blood.

Table 13.1 List of diseases transmitted through blood

VIRAL	BACTERIAL	PARASITIC
• Hepatitis B virus (HBV) • Hepatitis C virus (HCV) • Hepatitis G virus (HGV) • Human immunodeficiency virus (HIV) • Epstein Barr virus (EBV) • Cytomegalovirus (CMV) • Human T lymphotropic viruses (HTLV-1 and HTLV-II)	• Syphilis • Brucella	• Malaria • Toxoplasma • Microfilaria

13.2 TRANSFUSION ASSOCIATED HEPATITIS

Transfusion associated hepatitis (TAH) developing as a consequence of transfusion of infected blood or blood components affects every sphere of medicine. The illness initially often remains subclinical but could later result in chronic liver disease and even primary hepatocellular carcinoma. It is important that doctors working in transfusion medicine, general medicine, microbiology, laboratory medicine, pathology, social and preventive medicine should be aware of the spectrum and consequences of TAH.

Viral hepatitis being one of the serious complications of blood transfusion, can be due to different infectious agents like hepatitis A,B,C,D,E, and G visuses (HAV, HBV, HCV, HDV, HEV, HGV) besides cytomegalovirus (CMV) and Epstein Barr virus (EBV). Post transfusion hepatitis is mainly due to hepatitis B virus or hepatitis C virus. There are about three hundred million hepatitis B virus carriers and about one hundred million hepatitis C virus carriers in the world. Unfortunately most of the carriers of hepatitis viruses are not even aware of their carrier status.

13.2.1 Hepatitis A virus

Hepatitis A virus (HAV) was identified by Feinstone in 1973 and is primarily responsible

for infective hepatitis. Transfusion of Hepatitis A virus by blood transfusion is rare because of two main reasons:

- Presence of virus in blood for a short time.
- Rare carrier state.

Hepatitis A virus circulates in blood only during the initial acute phase of infection and during this period the individual is not the candidate for donation of blood. However, in rare cases viraemia maybe present before the development of symptoms and blood donated during this period could be an effective vehicle for transmission of HAV.

13.2.2 Hepatitis B virus (HBV)

Hepatitis B virus is the most important causative agent of TAH. Humans are the only reservoir of Hepatitis B virus (HBV). Hepatitis B virus was discovered by Blumberg et al in 1965 in the serum of an Australian aborigine and thus its antigen is also called as Australia antigen. The virus particle (virion) is a small complex double shelled structure having an external diameter of 42 nm with a nucleocapsid core and lipoprotein coat. This particle was first discovered by Dane and his colleagues in 1970 and thus is called Dane particle. It represents the complete HBV. The nucleocapsid core is 27 nm in diameter. It replicates in the nuclei of infected hepatocyts and possesses a distinct antigen called hepatitis core antigen (HBcAg). The virion core antigen contains DNA polymerase and double stranded DNA molecule **Fig. 13.1**.

The major structural protein is lipoprotein viral coat and is called hepatitis B surface antigen (HBsAg). This protein is synthesized in the cytoplasm of infected hepatocytes in excess quantities than that needed for viral assembly. The excess material in the form of small particles, either 22nm spherical particles or smaller tubular particles of varying lengths are released in the blood stream. Since these smaller particles contain only the major viral protein HBsAg they are considered non-infectious but act as markers of infection. Occasionally complete virus occurs in the blood stream. The virion has an other marker i.e. hepatitis B "e" antigen (HBeAg) which is closely related to core antigen and is considered as a marker of infectivity.

Subtypes of HBV: HBV has an antigenic heterogenicity. It consists of at least three different antigenic components: a group specific antigen **a** and two pairs of type specific

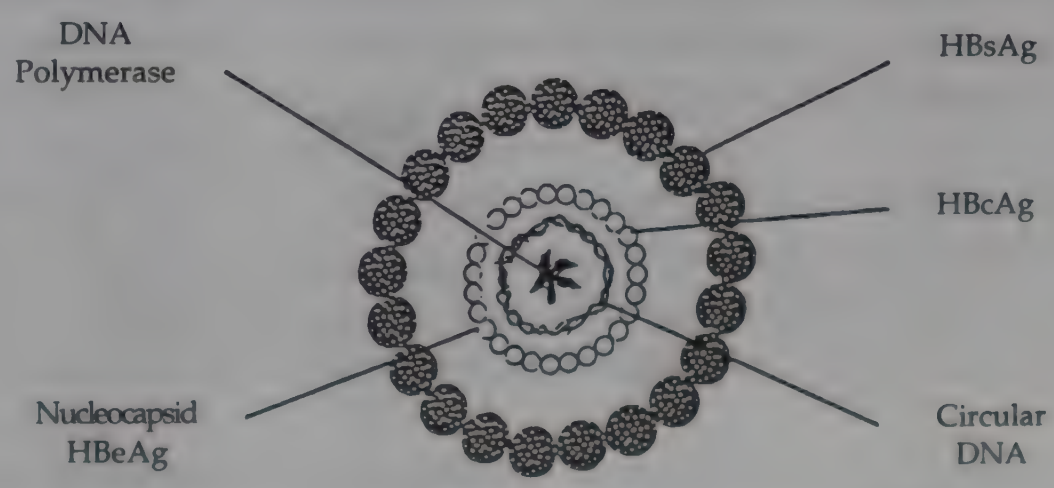


Fig. 13.1 Structure of HIV

antigens **d-y** and **w-r**. Only one member of each pair being present at a time. HBV can thus be divided into four major antigenic subtypes **adw, adr, ayw** and **ayr**.

Transmission of HBV infection: The virus is highly infectious and minute amounts of blood/body fluids as little as 0.01 µl can transmit the infection. Transmission of HBV is mainly by the parenteral route which involves direct contact with body fluids. The most common routes of infection are:

- Direct or indirect contact with infected blood (needle sticks, cuts etc.).
- IVDU (sharing needles).
- Unprotected sexual contact.
- Transfusion of infected blood or blood products.
- Vertical transmission (perinatal transmission).

Clinical presentation of HBV infection: The incubation period of HBV infection is about 50-150 days during which the patient has no signs and symptoms but the virus may be detected in the blood stream. There is a prodrome of malaise, weakness, loss of appetite, nausea followed by symptoms such as fever, rash, arthralgia and jaundice depending upon the severity of infection. In mild cases jaundice does not occur however, more severe case can result in serious disease. Antibodies to three main antigens i.e. anti-***HBsAb***, anti-***HBcAb*** and anti-***HBeAb*** appear at different times mainly during the course of disease and may persist for years, some times for life after recovery.

Anti-***HBsAb*** is usually associated with resistance to reinfection

Anti-***HBcAb*** is not protective but is considered as a sensitive indicator of HBV replication. Its presence in asymptomatic blood donor indicates either recent HBV infection or chronic carrier state.

Anti-***HBeAb*** is seen mostly in healthy carriers of HBsAg.

In 10 to 20% of clinically diagnosed HBV infections, the infection does not resolve but enters into chronic phase, which may last for just six months and then resolve or persist for life. In some chronically infected persons, no ill effects are seen but may develop chronic liver disease which may lead to cirrhosis, hepatocellular carcinoma and death.

Serological and biochemical markers of HBV infection: The development of various serological markers during the acute and chronic HBV infection are shown in **Fig 13.2** and **13.3**, respectively and the interpretation of various markers is given in **Table 13.2**.

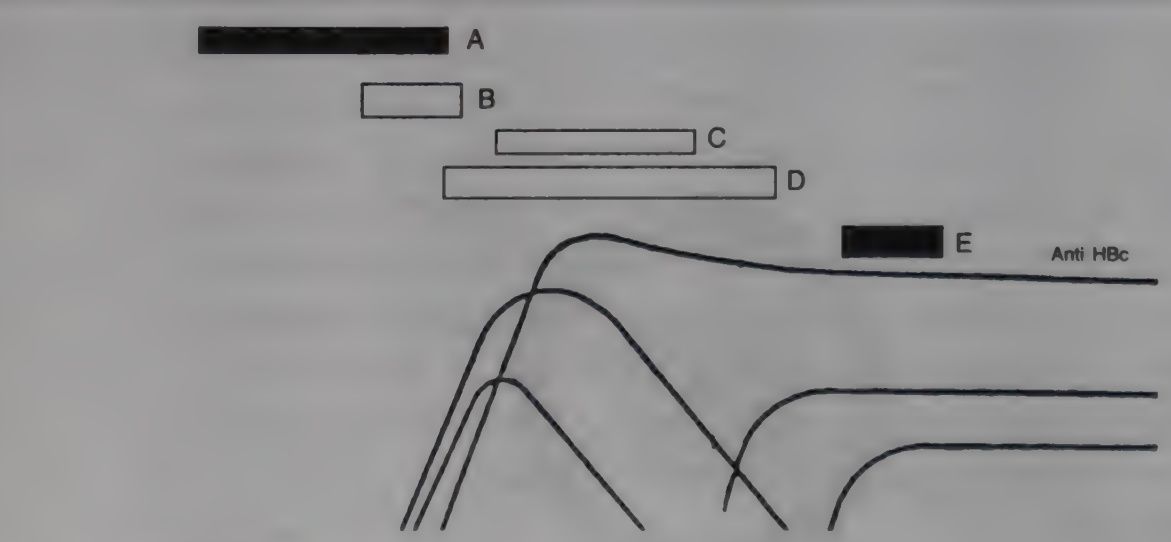


Fig. 13.2 Serological profile of acute HBV infection

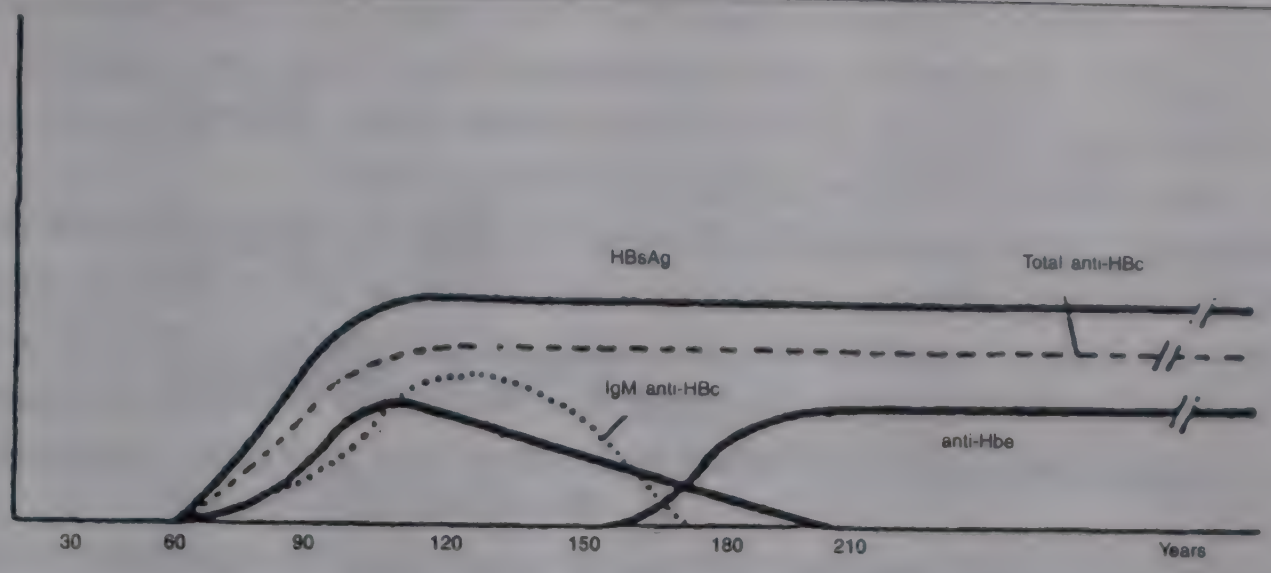


Fig. 13.3 Serological profile of HBV infection

Table 13.2 Interpretation of various HBV markers

HBsAg	HBeAg	anti-HBc	anti-HBe	anti-HBs	Interpretation
–	–	–	–	–	No evidence of present or past HBV infection.
+	–	–	–	–	Incubation period of HBV.
+	+	+	–	–	Early HBV infection with high infectivity.
+	–	+	+	–	Late acute HBV infection or chronic HBV infection with lower infectivity.
–	–	+	+	–	"Window" period in late acute HBV infection.
–	–	+	+	+	Convalescent from HBV infection.
–	–	+	–	+	Late phase of convalescence (anti-HBe has waned).
–	–	–	–	+	Response to HBV vaccine or recent administration of hyperimme anti-HBs immunoglobulin.

Appearance of HBsAg in patient's serum: It is usually the first detectable serological marker in acute HBV infection and remains detectable from few days to several months depending upon recovery of the patient. In some cases HBsAg persists for years. These are called carriers.

Appearance of Anti-HBc (HBcAb): In most cases HBcAb appears in the serum during the acute illness (preicteric phase) and persists for months.

Appearance of HBV "e" antigen and its antibody: Hepatitis B "e" antigen (HBeAg) appears in the serum almost at the same time as HBsAg but disappears in most cases within a few weeks. Its disappearance is followed by the appearance of anti HBe (HBeAb) in serum which lasts for several months.

Appearance of anti HBs (HBs Ab): Antibody to HBs Ag (HBsAb) appears after the disappearance of HBsAg and indicates recovery from acute illness. At times the appearance of HBsAb may be delayed for weeks after HBsAg becomes undetectable and during this period called core antibody window, anti HBc-IgM may be the only detectable marker of recent HBV infection.

Prevalence of Hepatitis B infection: The prevalence of HBsAg carrier rate varies from 0.01% to 30% from one country to another. In general, countries with low socioeconomic structure have higher HBsAg carrier rates and most of the industrialised and developed

countries have HBsAg carrier rate less than 1%. Depending upon the prevalence of hepatitis B in the general population, the International Task Force on hepatitis B has classified geographical areas into low (< 2%), intermediate (2 to 7 %) and high prevalence (>7%) regions. India falls in the intermediate prevalence region.

Screening tests for hepatitis-B virus: In order to reduce the risk of post transfusion hepatitis-B infection, HBsAg screening was first of all attempted by Okochi et al at the University of Tokyo hospital in December, 1968. Donated blood was screened by immunodiffusion techniques for the presence of hepatitis B surface antigen (HBsAg). Since then a number of test procedures have been developed to screen the blood for the detection of HBsAg.

Currently the most sensitive and widely used tests for detection of HBsAg are based on EIA/ELISA principle. In India, unfortunately, many blood banks especially those with little or no blood in the quarantine stage and having walk in donor programmes are compelled by the circumstances to use rapid screening tests for detection of HBsAg. As these test procedures are based on latex agglutination or chromatographic principles, whose sensitivity is low, a large number of HBV blood units are cleared for transfusion and thus the infection gets transmitted to the unlucky patients. Centers having a large blood collection use ELISA method for screening of blood for presence of HBsAg.

Table 13.3 Methods for the detection of HBsAg in order of sensitivity

Test	Sensitivity (minimum level of HBsAg detected)
• Immunodiffusion (I.D.) • Immunoelectro-osmopheresis (IEOP)	Low (> 100 ng/ml)
• Latex agglutination (L.A.) • Reverse passive haemagglutination (RPHA) • Immuno chromatography test (ICT)	Moderate (10-100 ng/ml)
• Enzyme Immunoassay (EIA/ELISA) • Radio Immunoassay (RIA)	High 0.5-10 ng/ml.

Enzyme Immunoassay (EIA/ELISA)

Principle: Enzyme immunoassay is based on sandwich principle involving the use of solid phase e.g. the well of microplate coated with antibodies of HBsAg (**Fig 13.4**). Test sample (serum/plasma) is added to the microwell and incubated. At the end of incubation period, the serum is washed off and conjugate i.e. monoclonal IgG conjugated to tracer enzyme is added and incubated. HBsAg if present in the sample, allows the enzyme conjugate to bind to solid phase and build sandwich **antibody-antigen-antibody** complex. Excess conjugate is washed away and chromogen substrate is added for enzyme action to produce a colour. The intensity of the colour is directly proportional to the enzyme activity which in turn is proportional to the HBsAg concentration in the sample or control. The resulting colour can be detected either visually or by ELISA reader.

There are a number of commercial kits (micro ELISA test) available for detection of HBsAg and it is recommended that the procedure provided by the manufacturers in their kit is followed.

Reverse passive haemagglutination assay (RPHA): Reverse passive

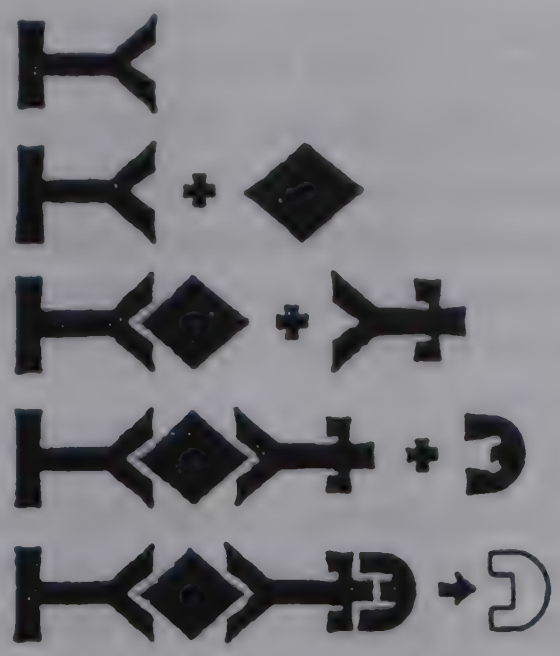


Fig. 13.4 Princple of ELISA for HBsAg detection

haemagglutination assay also gives satisfactory result but its sensitivity is lower than enzyme immunoassay so it is not in use presently.

Immuno chromatography test (ICT): It is a rapid strip test for the detection of HBsAg and is more sensitive than latex agglutination test.

Principle: In chromatographic immunoassay the membrane is precoated with anti HBsAg capture antibody on the test band region and goat anti-mouse antibody on the control band region. During testing the specimen (serum/plasma) is allowed to react with the colloidal gold particles which have been coated with anti HBsAg monoclonal antibody. The mixture then moves upward chromatographically by capillary action. In case of positive result a coloured band will be formed in the test band region of the membrane with specific anti HBsAg antibody colloidal gold particle complex. Absence of coloured band in the test band region indicates a negative result. In order to have procedural control, a coloured band will always appear at control region irrespective of the presence or absence of HBsAg.

Limitations of HBsAg screening: Even after using the most sensitive screening test for the detection of HBsAg on blood from voluntary donors, HBsAg negative units have been found to transmit the hepatitis B virus infection. The basic aim of blood transfusion services should be to provide zero risk blood for transfusion, however in actual practice this is not achievable beause of the following reasons:

- Lack of test which is 100% sensitive probably due to HBsAg mutants.
- Failure to detect HBsAg in some donors in the early incubation period.
- Difficulty in carrying out highly sensitive tests like DNA hybridization and PCR.

Extensive studies have shown that the exclusion of blood units with isolated high titre of anti HBc (even when HBsAg is negative) are effective in markedly reducing the chances of post transfusion hepatitis B infection. This fact led some of the blood banks to incorporate the testing for anti HBc in the donor screening protocols. This strategy is not adopted in our country and most of our blood banks restrict screening for hepatitis to HBsAg alone.

13.2.3 Hepatitis C virus (HCV)

HCV is the most common cause of post transfusion non-A non-B hepatitis in the developed world. The prevalence of HCV antibodies in blood donors in developed countries ranges from 0.4-2%. However in Egypt it is as high as 14%. The prevalence of HCV in Indian blood donors has not been studied extensively but as per some available reports the prevalence of anti HCV varies from 0.6% to 5.2% in voluntary/replacement donors. 50% of HCV infection is reported to progress to chronic infection of which 20% may progress to cirrhosis and hepatocellular carcinoma.

HCV was identified by cloning techniques. The virus is an enveloped single stranded RNA virus, 50-60 nm in diameter with a genome of 10,000 nucleotides and shares relationship with Flaviviruses. The genome encodes for three structural proteins; capsid protein (C-20KDa) and two envelope glycoproeins (El-gp35 and E2-gp70) and several nonstructural proteins (NS) i.e. NS2, NS3, NS4A, NS4B, NS5A, NS5B have been identified (**Fig. 13.5**).

The detection of antibodies to HCV is used for serodiagnosis of HCV infection. It was in 1990 that the **first generation test** for anti HCV was introduced which detected antibodies to C 100-3 and a part of NS4 region.

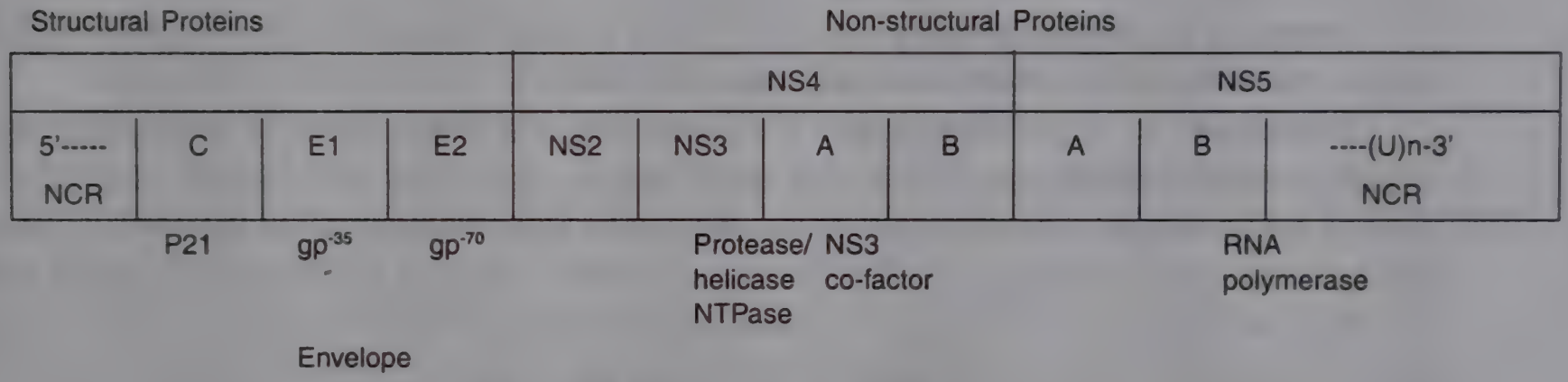


Fig. 13.5 Genome of HCV

The **second generation assays** were introduced in 1992 which included antigens from core and NS3 region (C22 and C200 from NS3-NS4/c33c fron NS3 alone) in addition to C100-3. **Third generation tests** have been developed which in addition contain antigen from NS5 region. The presently available recombinant immunoblot (RIBA) for the detection of antibodies to HCV includes bands 5-1-1, C100-3 C22-3, c33c and NS5 polypeptide. Recently kits have been developed which incorporate synthetic peptides instead of recombinant peptides marking the test more specific. If antibodies to 2 or more antigens are present, the sample is considered as positive. Prior to the development of specific markers certain Surrogate markers i.e. determination of serum alanine aminotransferase (ALT) was used to identify the high risk donors for transmission of HCV. Although the **surrogate markers** played an important role in the prevention of hepatitis C prior to the introduction of HCV specific assays but presently the value of these tests remains inconclusive and controversial.

13.2.4 Hepatitis D

It is also known as Delta hepatitis. It is caused by a defective RNA virus that is unable to produce its own protein coat and thus coats itself with HBV surface antigen. The delta virus is dependent on pre-existing or concomitant HBV infection for propagation. Co-infection

causes acute viral hepatitis which often becomes fulminant and is usually fatal, while superinfection causes acute hepatitis progressing to chronic active hepatitis and cirrhosis in 75% of carriers.

Delta hepatitis has parenteral mode of transmission and usually follows HBV infection. Although tests for delta antigen and antibody exist but should not be used in blood donor screening as all infections with delta virus are positive for HBsAg. So routine testing for HBsAg will eliminate the risk of transmission of Delta virus.

13.2.5 Hepatitis G virus (HGV)

Hepatitis G virus is an enveloped RNA virus and belongs to the Flaviviridae family. The virus was discovered in 1995. The virus genome is similar to that of HCV with which it shares a 25% homology at the nucleotide level. The HGV appears less variable although variants have been described. HGV replicates in peripheral blood cells but its replication in the liver is still questionable. The mode of spread of HGV is almost same as HCV. The HGV can be detected in the serum by nucleic acid technology (PCR). Recently an immuno-assay has been developed to detect antibody to HGV.

The prevalence of HGV in blood donor population by doing antibody tests in the developed countries ranges between 1 to 5% but the detection of antibody generally indicates recovery from and probably the immunity to the HGV agent.

Prevention of post transfusion hepatitis: Following points should be kept in mind for prevention of post transfusion hepatitis:-

- Promotion of voluntary blood donation.
- Proper donor interview and selection.
- Use of most sensitive and specific test for HBs Ag detection (3rd generation tests).
- Screening for antibody to hepatitis core antigen (Anti-HBc) if possible.
- Screening for anti- HGV to detect carrier of HGV is preferable.
- Good manufacturing practice (GMP) for blood fractionation and incorporation of virucidal technology (Solvent detergent technique) for viral inactivation.

13.3 MALARIA

Transmission of malaria can occur after transfusion of whole blood, red cells or any blood component contaminated with red cells. Plasma fractions do not transmit the disease. The parasites of all species remain viable for at least one week in stored blood at 2-6°C. However, Plasmodium falciparum can remain viable for 2-3 weeks. The incubation period of Plasmodium vivax and Plasmodium falciparum is one week to one month while that of Plasmodium malariae is many months. Post transfusion malaria (PTM) is particularly dangerous in pregnant women and immunodeficient patients.

13.3.1 Detection of malaria in carriers

Examination of blood film: The examination of blood film is not suitable for screening large number of blood donations because it is difficult to find parasites in blood film in the short available time especially if density of parasites is less than 100 per microlitre of blood. However, the examination of blood films for parasites within red cells is considered best

method for diagnosis in suspected cases.

Test for malaria antibody: Tests for malaria antibody either by indirect fluorescent antibody (IFA) test or ELISA are alright for nonendemic areas but not ideal for malaria endemic areas e.g. India as there is a likely chance that many blood donors will have antibodies and will be rejected.

13.4 CYTOMEGALOVIRUS (CMV) INFECTION

Cytomegalovirus may be transmitted by blood transfusion and can cause severe disease in seronegative premature babies of low birth weight and seronegative immunosuppressed patients e.g. patients of bone marrow transplantation or patients with HIV infection.

Since more than 50-80% of adults in developed countries and almost 100% in some developing countries have antibodies to CMV, so screening of all donors is not practically feasible. In high risk recipients it is advisable to use leucocyte depleted blood.

13.5 SYPHILIS

Syphilis is a disease caused by Treponema pallidum. It is primarily a sexually transmitted disease but the organism can gain entrance in to the body through minute lesions on skin or mucous membranes.

Blood transfusion is a potential route of infection, particularly if fresh infected blood is transfused. The incubation period in transfusion Syphilis is on the average 9-10 weeks and recipient usually presents with secondary eruptions.

The chances of post transfusion Syphilis have been greatly reduced because of the following reasons:

- Exclusion of high risk donors.
- Screening for Syphilis.
- Storage of blood at 2-6°C for 72 hours prior to transfusion. (Spirochaetes are very sensitive to temperature and die at lower temperature).

13.5.1 Serological test

There can be non specific tests and specific tests.

Non Specific test: The non specific tests include:

- VDRL (Veneral Diseases Research Laboratory test)
- RPR (Rapid Plasma Reagin) test.

VDRL test: VDRL test is a simple, rapid and economical test for screening syphilitic infection, however the test needs inactivation of the specimens (sample) and the shelf life of the reconstituted antigen is poor.

RPR test: It is a macroscopic non-treponemal flocculation test for screening syphilitic infection. The test is rapid and does not need inactivation of sample, the antigen used is a modified VDRL antigen (cardiolipin) which contains microparticulate charcoal/carbon to enhance the visual interpretation. The test is supplied in the form of kits by manufacturers and it is advised to follow the instructions provided with the kit.

Specific tests: Specific tests are mainly used for confirmation and are not suitable for routine screening.

- Treponema pallidum haemagglutination test (TPHA).
- Fluorescent treponemal antibody test (FTA)
- Enzyme immuno assay (EIA).

13.6 CREUTZFELDT-JAKOB DISEASE (CJD)

Creutzfeldt-Jakob disease is one of the series of transmissible spongiform encephalopathies (TSE) and is thought to be caused by a proteinaceous particle smaller than a virus. This nucleotide infectious particle is called Prion which was once thought to be a slow virus. Although transmission of CJD through blood and blood products has been speculated but no case has been reported.

13.7 HUMAN T-CELL LYMPHOTROPIC VIRUSES

Human T-cell lymphotropic virus type-I (HTLV-1) was the first human retrovirus isolated from a patient with adult T-cell leukaemia/lymphoma (ALT) by Robert Gallo and his group in 1980. The disease can persist for a life time but usually does not cause major illness in most people who are infected. In rare instances HTLV-1 is associated with a chronic degenerative demyelinating neurological disease called tropical spastic paraparesis (TSP) but now often called HTLV associated myelopathy (HAM). HTLV-1 is more common in southwestern Japan and Caribbean islands. The route of transmission for HTLV-1 is by sexual contact (predominantly male to female), by parenteral exposure to blood and mother to child through breast milk.

The second human retrovirus HTLV-II was discovered several years after HTLV-1. It is closely related to HTLV-1 and it is difficult to differentiate between these two viruses in the routine laboratory. Although no firm association between HTLV-II and any disease has been found but the viurs was first found in a patient with hairy T-cell leukaemia and subsequently in other T-cell malignancies, viral RNA expression has not been found in malignant cells. High prevalence of HTLV-II has been seen in I.V. drug abusers and their sexual contacts in USA and other countries. The prevalence of HTLV-I and HTLV-II in intravenous drug abusers in New York is 9% and 18%, respectively. The prevalence of HTLV-I and HTLV-II in India is not available.

The viruses are transmitted by transfusion of cellular components of blood but not by cell free components like plasma. Universal screening of blood donors for HTLV-I was introduced in December, 1988 in USA and in July, 1991 in France. In south western Japan where HTLV is, endemic screening of blood markedly reduced the rate of seroconversion in recipients of blood.

Several HTLV screening assays are available like gelatin particle agglutination (P.A.), enzyme immunoassay (EIA) and indirect immunoflorescence (IF). The sensitivity and specificity of ELISA tests ranges from 98.4% to 100%. None of the screening tests available is able to differentiate between anti HTLV-I and anti HTLV-II because of cross reactivity due to 75% homology of amino acid sequences between the two viruses. Confirmatory assays like western blot, RIPA, antibody capture radioimmuno assay and PCR are also available.

Chapter 14

INFECTIONS TRANSMITTED BY TRANSFUSION OF BLOOD COMPONENTS AND BLOOD PRODUCTS

14.1 INTRODUCTION

The transfusion of blood began with the use of whole-blood and continued for many years. However, nowadays a variety of blood components and products are available for their use in patients of anaemia, thrombocytopaenia, neutropaenia, abnormal coagulation and immunodeficiency, etc. Whole blood is transfused mainly in cases of rapid blood loss due to massive uncontrolled haemorrhage etc. The term blood-component is used for those blood materials that are separated by means of differential centrifugation followed by expression into satellite/secondary packs attached to the original donor blood pack whereas blood products are those blood materials that have been extracted and purified from crude plasma and preserved in stable form. Some of the important blood products and blood components have been summarized in **Table 14.1**. These products are supposed to be life saving drugs. However, unfortunately these blood products/components are associated with risk of transmission of viral, parasitic and bacterial diseases. Etiological agents of these various diseases, listed in **Table 14.2**, have got excellent opportunity to enter in a new human host. Out of the total adverse effects of transfusion of blood/products/components approximately 40% are due to transmission of these etiological agents. The degree of problem caused by these microbial agents is directly related to their endemicity in a particular area.

Table 14.1. List of various types of blood components/products

Cellular components	Blood components	Blood products
• Red blood cells • Leucocyte reduced red cells • Washed red blood cells • Frozen/deglycerolysed red cells • Platelet concentrate • Granulocyte concentrate	• Single donor plasma • Fresh frozen plasma • Cryoprecipitated antihaemophilic factor	• Factor VIII concentrate • Factor IX concentrate • Albumin Plasma protein fraction • Immune serum globulin • Rh D immunoglobulin • Antithrombin III concentrate • Hyperimmune serum

14.2 MICROBIAL AGENTS TRASMITTED THROUGH TRANSFUSION OF BLOOD PRODUCTS AND COMPONENTS

14.2.1 Viruses

Maximum percentage of transfusion transmitted diseases are viral followed by parasitic and bacterial infections. Transmission of viruses is very high if the blood/components/products are from large pool of plasma taken from many donors. The transfusion transmissible viruses are listed in **tables 14.2** and **14.3**.

Table 14.2. Microbial agents transmitted through blood products

Viruses	Parasites	Bacteria (can contaminate the product after or during processing)
• HIV-1 and HIV-2 • Hepatitits B virus • Delta virus • Hepatitits C virus • Hepatitits G virus • Hepatitits A virus • Cytomegalovirus • Epstein barr virus • Human parvovirus B-19 • HTLV-I • HTLV-II • Transfusion transmitted virus (TTV)	• Plasmodium spp. • Microfilaria • Toxoplasma gondii • Babesia microti • Leishmania spp. • Trypanosoma cruzi.	• Aerobactor • Achromobacter • Alcaligenes • Brucella spp. • Staphylococcus albus • Staphylococcus epidermidis • Haemolytic Esch. coli • Pseudomonas • Enterobacter • Klebsiella • Yersinia • Proteus

Table 14.3 Transmission of viruses by plasma/cellular components

Plasma	Cellular components
• HIV-1 and HIV-2 • Hepatitits B virus • Hepatitits C virus • Hepatitis G viruse • Delta virus • Hepatitits A (rarely) • Parvovirus	• HIV-1 and HIV-2 • Human T-lymphotropic virus HTLV-I • Human T-lymphotropic virus HTLV-I • Epstein Barr virus

14.2.1.1 Retroviruses

Retroviruses were known to cause malignancy and leukaemia in animals and were never suspected to be a cause of human disease. The advancement in molecular biology and recombinant DNA technology led to the discovery of the first human T lymphocyte virus-Type HTLV I. Subsequently HTLV II was also discovered. The knowledge about these viruses encouraged the workers to prove the etiology of AIDS virus. The AIDS virus was initially named as LAV or HTLV III. Retroviruses responsible for AIDS are primarily of two types HIV-1 and HIV-2, these have been discussed in chapters 6 and 7.

The first report of transmission of AIDS by factor VIII concentrate was published by CDC Atlanta in USA. Blood transfusion is a very efficient mode of transmission for HIV, as more than 90 percent recipients of blood from HIV seropositive donor, develop infection and AIDS. Transfusion induced infection may lead to AIDS more rapidly than the infection transmitted by other routes/modes. Fortunately transfusion associated HIV infection is decreasing because of the prior screening of blood products. There is a possibility of transmission of HIV through blood and blood products taken from donors during window period when antibodies to HIV are not detectable.

HTLV I and HTLV II are considered to be oncoviruses. These viruses are lymphoproliferative and cause malignancy. These viruses are transmitted much in the same way as AIDS viruses. The first detailed report of transfusion associated HTLV I transmission was from Japan in 1980s. These viruses are transmitted by transfusion of cellular elements of blood and not by cell free components/products. Seropositivity is found within two months of exposure.

Both HTLV I and HTLV II are single stranded RNA viruses. These are sensitive to organic solvents, detergents and heat but resistant to UV light and X-rays. Its genome codes for viral coat protein in the gag-region, polymerase in the pol region and surface envelope protein in the envelope region. The virus binds the helper/inducer T cells through CD4 receptors. It may remain latent in the infected cell for a long time.

Screening tests for HTLV: Several HTLV assays are available like gelatin particle agglutination (PA), enzyme immunoassay (EIA) and indirect immunofluorescence (IF). The sensitivity and specificity of ELISA ranges from 98.4% to 100%, respectively. None of the screening tests available is able to differentiate between anti HTLV-I and anti HTLV-II because of cross reactivity due to the 75% homology of amino acid sequences between the two viruses. Confirmatory assays like western blot, RIPA, antibody capture radioimmuno assay and PCR are also availabe.

14.2.1.2 Cytomegalovirus (CMV)

CMV is one of the largest Herpes virus with double stranded DNA. This infection is wide spread and is transmitted by a number of well-recognized routes. The infection can also be transmitted by organ transplantation and transfusion of blood/blood products. Transmission of CMV by blood was first reported in 1966. It is presumed that CMV is transmitted by cellular blood components.

After transfusion the virus gets reactivated. The infection can be severe in immunocompromised patients and premature infants. It may be fatal in cases of transplantation of allogenic marrow. Patients of renal and heart transplantation may develop severe primary CMV infection followed by transfusion of contaminated blood/cellular components.

Transfusion transmitted CMV can be checked/controlled to a greater extent only by depleting the white cells by filtration or centrifugation. At present the only method to avoid its transmission is by using the CMV negative donors. IgM specific anti-CMV ELISA can be used to screen the plasma products.

14.2.1.3 Parvovirus

Parvo virus B-19, a single stranded non enveloped DNA virus was discovered in 1975 in the serum of normal blood donors. It is a common cause of a blood disease known as erythema infectiosum and may be implicated in plastic anaemias. This clinical entity may be severe in pregnant women, AIDS cases, and haemophiliacs etc. Fortunately this virus is not a problem in India. Diagnostic methods like haemagglutination, gel test, DNA hybridisation and PCR have been developed.

14.2.2 Parasites

Most common parasites which may be transmitted through blood/blood products are malarial parasite and Toxoplasma gondii. Rarely, others like Leishmania spp. and Babesia microti etc. may also be transmitted through transfusion of blood/blood products.

14.2.2.1 Malarial parasites

Malaria is a major public health problem throughout the world. The infection is caused by a protozoan parasite of the genus Plasmodium. Four species of Plasmodium namely *P. vivax, P. falciparam, P. malariae* and *P. ovale* are responsible for human malaria.

The parasite can survive in the blood of host for many years followed by relapse or recrudescence. Such cases as blood donors pose potential threat of post transfusion malaria. Transfusion transmitted malaria can be lethal. Quite a good number of deaths have been reported due to transfusion transmitted malaria. To avoid the transfusion transmitted malaria, proper scrutiny and screening of blood donors should be done. Immunofluorescence method for detection of malaria has been found to be better than the routine microscopy method. Now enzyme based immunodiagnostic assays are available commercially as kits for early diagnosis and screening of blood.

14.2.2.2 Toxoplasma gondii

Toxoplasmosis is caused by an obligate intracellular protozoan parasite known as Toxoplasma gondii. The disease is prevalent throughout the world. Generally it is transmitted by ingestion of oocyst/cyst of parasite. The infection can also be transmitted by trophozoites in blood through transfusion. The infections initiated by oocyst or cyst are invariably mild but infection induced by trophozoites of blood stage is more severe and in the acute form. That trophozoite of parasite can survive in blood stored at 4°C upto 50 days. Leukocyte transfusion has also been associated with transmission of Toxoplasmosis in immunocompromised patients. Both these observations suggest that now transfusion associated Toxoplasmosis is posing a serious threat to AIDS patients.

14.2.3 Bacteria

Unlike the viruses the direct transmission of bacteria from donor to recipient is less common. Treponema pallidum has been found to be associated with transfusion induced syphilis. It is discussed in Chapter 13.

14.2.3.1 Bacterial contamination of blood/blood components/blood products

Handling and storage of blood, its components and products at room temperature as well as at 4°C may provide sufficient time and opportunity for bacterial contamination. The bacteria that have been found to contaminate the blood components and products are Aerobacter, Achromobacter, Alcaligenes, Brucella spp., Staphylococcus albus, Staphylococcus epidermidis, haemolytic Esch. coli, Pseudomonas spp., Enterobacter spp., Klebsiella spp., Yersinia spp. and Proteus etc.

Transfusion of blood contaminated with bacteria has a typical clinical syndrome of violent chills during the transfusion or shortly after the transfusion. The symptoms may be due to endotoxins. Sometimes transfusion of contaminated blood may cause septic shock syndrome resulting in death of the recipient. Bacteria commonly associated with septic shock are Esch. coli, Enterobacter spp., Pseudomonas, Klebsiella spp. etc. Septicaemia has been reported following the transfusion of plasma and cryoprecipitates etc.

In most of the cases, contamination of blood, its components/products occurs due to the introduction of bacteria at the time of collection, processing, manufacturing or storage. With the improvement in collection and storage methods, the contamination of blood with bacteria has declined significantly. The blood contaminating organisms are mainly ubiquitous Gram negative bacteria. In blood they grow very fast and unfortunately the contaminated blood may appear normal even with heavy contamination, as these bacteria are not haemolytic. Gram positive bacteria which form a part of skin flora (Staphyloccocus and diphtheroids) are rarely involved in transfusion associated diseases.

Apart form blood, which is a good culture medium for micro-organisms the citrate, glucose and anticoagulant preservative solutions are ready made energy source for these bacteria. Fortunately, macrophages and leukocytes present in blood have natural bactericidal capacity.

Bacterial contamination of blood/products/components can be prevented to a certain extent by:

- Use of closed blood collection systems
- Transfusion of blood within 6-8 hrs. of collection or removal from refrigeration.
- Preparation of blood components in sterile conditions.
- Proper inspection of blood unit for haemoloysis/discolouration of RBCs.

14.4 PREVENTION OF TRANSFUSION ASSOCIATED DISEASES CAUSED BY BLOOD PRODUCTS/COMPONENTS

Blood products/components have been recognised as life saving drugs by WHO. A wide range of blood components and blood products are now available that are being manufactured throughout the world. Till 1980s the major concern of transfusion induced diseases was only hepatitis viruses but the detection of AIDS virus alerted the entire world to combat the menace of transfusion associated infections.

For controlling the transfusion associated transmission of infections quality control/ quality assurance ; good laboratory practices (GLP), good manufacturing practices (GMP) including virucidal technologies and biosafety procedures etc. should be implemented strictly.

14.4.1 General control measures:

- Transfusion should be given only when there is a clear medical indication.
- Screening of units of donated blood and products for all the diseases notified as already discussed in Chapters 6, 7 and 13 (HIV; hepatitis, Malaria and Syphilis).
- Autologous transfusion should be encouraged.

14.4.2 Good laboratory practices/good manufactering practices

To control such transmission following measures should be taken. Transmission of transfusion associated infections can be effectively controlled by effective implementation of Quality Control/Quality Assurance programme.

- Selection of Normal Healthy Donors.
- Immunodiagnostic kits/reagents for diagnosing the transfusion associated diseases

must be validated by Quality Control Authority/Regional labs.

- Strict GLP/GMP should be followed.
- All the equipments/instruments used for transfusion must be sterile.
- Transfusion committee must be operational and should have written quality plan for transfusion safety and standard operative procedures (SOPs) for various tests etc.
- Prevention of bacterial contamination during donation, collection, storage and processing of blood.
- Effective viral inactivation of certain plasma products/components.
- Judicious use of blood/blood products/components.
- Quality audit.
- Enforcement of corrective measures.
- Biological safety cabinet should be used for handling the biological materials.
- Proper handling and disposal of disposables like needles, syringes and bags should be ensured.
- Effective decontaminants should be used for cleaning/decontamination of work place.
- Blood should be centrifuged in covered containers.
- Equipments should be decontaminated before/after repair/transfer etc.
- Protective clothings (gown, mask etc.) should be used.
- Hands should be washed properly.

14.5 QUALITY CONTROL

14.5.1 Quality control of equipments and immunodiagnostic kits/methods

The instruments under use for storage, handling, processing, testing etc. must be properly maintained and monitored to ensure Good Laboratory Practices and Good Manufacturing Practices (GLP/GMP). Biosafety cabinet, automatic cell washers, water bath etc. must be validated.

Immunodiagnostic kits/reagents and methods used for screening of the donor must be evaluated for QA/QC for assuring the proper diagnosis of diseases.

14.5.2 Quality control of personnel

Quality control of personnel is one of the most important aspects of quality control programme as it is central point around which the entire process revolves. Personnel also regulate the entire process of QA/QC.

All personnel should be:

- Trustworthy.
- Properly qualified/trained and able to withstand stressful situations.
- Acquainted with SOPM.
- Tested with proficiency testing.
- Should be trained regularly through continuing medical education programmes.
- Should be given suitable incentive/commendation from time to time.

14.5.3 Quality control of blood components/quality control of procedures

Quality Control Programme should be strengthened in GLP/GMP which consist of (i) collection of blood (ii) testing (iii) separation for components (iv) preparation of blood products (v) viral inactivation (vi) proper storage (vii) safe transfusion in patients and (viii) impact assessment.

There are six important steps of quality control in transfusion medicine services.

- Immunohaematological investigations.
- Microbiological investigations.
- Blood collection
  a) Donor recruitment
  b) Donor's investigations
- Separation of blood components and preparation of blood products.
- Storage.
- Use of associated equipments.

Chapter 15

COUNSELLING AND HIV/AIDS TESTING

15.1 DEFINITION

Counselling is a process where one person, explicitly and purposefully, gives his/her time, attention and skills to assist a client to explore their situation, identify and act upon solutions within the limitations of their given environment. In simple terms, counselling is a process in which two people meet and have a dialogue to resolve a crisis, solve a problem or make decisions involving highly personal and intimate matters and behaviours.

World Health Organisation (WHO) defines HIV/AIDS counselling as a dialogue between a client and a care provider aimed at enabling the client to cope with stress and to take personal decisions relating to HIV/AIDS. The counselling process includes the evaluation of personal risk of HIV transmission and the facilitation of preventive behaviour.

15.2 DIFFERENCE BETWEEN COUSELLING AND HEALTH EDUCATION

Counselling differs from health education in many ways and they are:

- Counselling is usually an 'one is to one' process whereas, health education addresses a group of people.
- Counselling is useful for not only giving information but also for changing attitudes and motivating behavioural change. Health education is used mostly for information sharing.
- Counselling sessions involve personal problem solving. In health education, general issues are discussed.
- Counselling is more focussed, specific and goal targeted, whereas health education is much more generalized.
- Counselling evokes strong emotions in both counsellor and client whereas health education sessions are emotionally neutral in nature.

15.3 OBJECTIVES OF HIV/AIDS COUNSELLING

The objectives of HIV/AIDS counselling include:

- Prevention of HIV infection by changing life styles and behaviours. (Primary prevention).
- Providing psychological support to those already infected.
- Motivating for change in high risk behaviour (Secondary prevention).

15.4 WHY IS HIV/AIDS COUNSELLING NECESSARY

HIV/AIDS counselling is mandatory for providing voluntary HIV testing services. This is so because diagnosis of HIV in an otherwise healthy individual induces a series of psychological reactions like denial, anger, anxiety, depression, to finally acceptance. So far, there is no successful cure or vaccine available for HIV infection. In order for the individual

to be able to accept the infective status, carry on with life, plan future, prevent transmission and continue to function as a useful member of community, counselling is a must. Counselling induces a positive attitude and high life force in the individual helping him to carry on as before inspite and irrespective of the HIV infection, HIV/AIDS counselling is mandatory (pre and post test) as laid down by WHO/UNAIDS. Government of India is also actively emphasising the necessity of HIV counselling.

15.5 WHO REQUIRES HIV/AIDS COUNSELLING?

HIV/AIDS counselling is recommended for the following groups of people:

- Persons already identified as having AIDS or being infected with HIV and their families.
- Those being tested for HIV (pre and post test).
- Those seeking help because of past or current risk behaviour and planning their future.
- Those not seeking help but practicing high risk behaviour.

15.6 WHERE CAN HIV/AIDS COUNSELLING BE PROVIDED?

HIV/AIDS counselling can be provided in any setting including hospital wards, STD clinics, ANC clinics, family planning clinics, blood donation centers, drug deaddiction centers, prisons, primary/secondary health centers and community based programmes.

15.7 WHO CAN PROVIDE HIV/AIDS COUNSELLING?

HIV/AIDS counselling can be provided by anyone who has a sympathetic ear, can give time to listen, has knowledge of accurate scientific facts about HIV/AIDS and undergoes systematic and periodic training in counselling. In addition to doctors, nurses, psychologists, psychotherapists and social workers, even teachers, community and peer leaders, youth and self help groups can undertake preventive and supportive counselling.

15.8 PRE-REQUISITES OF COUNSELLING

Some of the pre-requisites of counselling include:

- Time and availability of counsellor
- Acceptance of the activity by counsellor
- Easy accessibility to counselling
- Aptitude for maintenance of confidentiality
- Correct knowledge and information about HIV/AIDS and policies etc.
- Consistency in counselling

15.9 TYPES OF COUNSELLING

- Pre-test
- Post test-positive, negative and indeterminate results.
- Follow-up

- General
- Family

15.10 CONTENTS OF PREVENTIVE HIV COUNSELLING:

- Determine whether the behaviour of an individual or group of individuals involves a high risk behaviour which can lead to HIV infection.
- Work with individuals to make them understand the risks and acknowledge the risks associated with their behaviour.
- Define and discuss with them how their life, attitude/values and self image is linked to these behaviours.
- Help individuals to define their potential for attitude shifts, behaviour modification and change.
- Work with individuals to introduce and sustain the modified behaviour.

15.11 COUNSELLING

HIV/AIDS is a life long disease which to date, has no cure or vaccine. In addition, lack of a healthy non judgmental and non discriminatory environment has resulted in isolation, victimisation and breach of fundamental rights including denial of basic medical services to people with HIV infection. HIV/AIDS counselling can help people in accessing correct information, assessing risks, making appropriate behavioural changes, leading to protection of self and others. This will help in developing coping mechanisms in people with HIV infection. Counselling can lead to empowerment and raise individual consciousness and can make individual take responsibility for their own behaviour.

Chapter 16

INFRASTRUCTURE FOR HIV TESTING LABORATORIES

16.1 INTRODUCTION

Establishment of optimal infrastructure for HIV testing laboratories is one of the most important components to ensure quality testing. However, the specifications regarding the required infrastructure cannot be made exclusive and rigid in a developing country like India with limited resources. For instance, the same ELISA reader and washing system may be utilised for screening both HIV and HBV within the common space and by the common manpower (e.g. technician). The optimal infrastructure for different categories of HIV testing laboratories is described here.

16.2 CATEGORIES OF HIV TESTING LABORATORIES/CENTRES

HIV testing laboratories have been categorised into following major groups by National AIDS Control Organisation, GOI.

16.2.1 Screening laboratories in blood banks

The main function of these laboratories is to ensure donated blood safety. Thus the laboratories in blood banks in addition to other testings e.g. grouping, cross matching must screen the donated units of blood for transfusion transmitted infections (TTI) i.e. HIV, HBV (HBsAg), syphilis and malaria as per the existing guidelines from GOI. These laboratories are also called "Zonal Blood Testing Centres" (ZBTCs).

16.2.2 Sentinel surveillance laboratories and voluntary testing laboratories

These categories of laboratories are functioning in various medical colleges, hospitals and institutes. These laboratoires carry out voluntary testing (with pre and post test counselling) and testing for surveillance. Being located in medical colleges, hospitals and institutes, the necessary infrastructures for HIV testing in these laboratories are likely to be in place.

16.2.3 Reference centres

Presently there are 9 referral centres distributed all over India that are entrusted with the following broad responsibilities:

- Providing referral HIV diagnostic services in the region.
- Evaluation of HIV test kits.
- HIV screening of blood products for human use.
- Carrying out external quality assurance of other laboratories and trouble shooting.

- Providing training in HIV testing and biosafety procedures.
- Providing technical support for formulating guidelines for quality assurance and other HIV testing related issues.
- Voluntary testing with counselling.
- Providing HIV testing services for various groups as directed by NACO, G.O.I. from time to time.
- Providing diagnostic services (including training) for opportunistic infections.

In India, the above three categories are already functioning, but with varying degrees of infrastructure. The various levels of infrastructure necessary for the various categories of HIV testing laboratories/centers are mentioned in **Table 16**.

16.2.4 Laboratories monitoring the progression of HIV infection

These laboratories carry out tests which can accurately assess the stage of HIV infection in an individual as well as predict its course and monitor its progression. These tests are valuable during the period of clinical latency in monitoring disease progression and evaluating response to antiretroviral therapy.

The details of the investigational setups necessary for monitoring the stage of disease in HIV infected individuals have been discussed in details in Chapter 12. The main equipments/facilities required are:

- Flow cytometry set up for – Enumeration of peripheral lymphocyte subpopulations. (specially CD4 lymphocyte counts)
- PCR set up for – Quantitating viral load
- Diagnostic kits for – p24 antigen assay
  – Serum β-2 microglobulin assay
  – Serum α-neopterin assay
- Skin testing panel antigens and applicators for – Delayed cutaneous hypersensitivity testing.

16.2.5 Diagnosis of opportunistic infections

Most of the infrastructure necessary for diagnosis of spectrum of opportunistic infections associated with HIV/AIDS are available in a multidisciplinary hospital. Some of them that are relevant for HIV/AIDS patients are mentioned below:

- Routine virology laboratory.
- Immunofluorescent microscopy facilities and necessary reagents.
- Bacterial culture (anaerobic and aerobic).
- Tuberculosis laboratory.
- Routine histopathology set up.
- X-ray equipment, film and reagents for film processing.
- Equipment for ultrasonography, bronchoscopy, endoscopy.
- MRI equipment, CT equipment.
- Ophthalmoscope.

Since, this document is on HIV testing the details of infrastructures for above mentioned laboratories have not been included in this document.

Table 16. Necessary infrastructure for various categories of HIV testing laboratories/centres

Category of HIV testing laboratories/ centre	Space*	Manpower	Equipments and supplies	Comment
1. Blood Bank	Two rooms (approximately 12 x 12 ft.) Room 1 for - putting up the test - storage of samples - placement of equipments - processing of samples - record keeping Room 2 for - decontamination and disposal of laboratory wastes.	Supervising Medical Officer -1 Technician/Laboratory assistant - 1 Laboratory attendant cum-cleaner - 1	**Biosafety:** Lab. coats/gowns (front closed) Chappals/slippers (top covered) Gloves Sodium hypochlorite/ethanol/ glutaraldehyde Discarding jars Disposable absorbent paper sheet (for spreading on the working area) Disposable bags (for carrying infectious wastes) Autoclave, preferrably incinerator Hot air oven. Needle destroyer/shredder **Collection of samples:** Screwcapped leak proof, nonbreakable tubes Centrifuge for separation of serum **Laboratory testing:** Water bath (35-44°C) Incubator BOD (35°-44°C) Balance (physical) Balance (electrical) Rocker (40-60 rockings/minute) ELISA reader ELISA washing system i.e. automatic ELISA washer Filters for ELISA reader (405 nm, 450 nm, 492 nm, 540 nm) ELISA paper role Glass pipettes 2ml, 5ml, 10 ml Microtips (capacity 25 ml, 50 ml, 100 ml) Microtip container Multichannel pipettes (50-200 ml)	For activities other than HIV testing in blood banks additional infrastructure will be necessary.

			Micro pipettes (5-50 ml, 50-200 ml, 200-1000 ml) Pipette stands Pasteur pipette, rubber teats Plastic forceps, plastic troughs Tissue paper HIV screening test kits (ELISA/rapid) **For storage of kits and reagents:** Refrigerator (domestic with freezer compartment) Deep freeze (- 20°C) Plastic storage vials Racks for holding storage vials	
2. Voluntary testing laboratories/ sentinel surveillance laboratories	**Two rooms as in 1 above plus** an additional room for - sample receiving and processing (Total number of rooms = 3)	**As 1 above plus** Counsellor - 1 Field worker -1 (for collection of samples etc.)	**As for 1 above plus** For collection of samples - Disposable syringes (2ml, 5ml, 10ml,) or vacutainers with gel - Disposable needles (21 gauge) **For Referral** - Sample carrier (e.g. thermocol box)	
3. HIV reference laboratories/ centres	**Three rooms as in 2 above plus** an additional room for- Counselling (Total number of rooms = 4)	**As as in 2 above plus** Supervising Medical Officer -1 Technician - 1 Laboratory assistant - 2	**As for 2 above plus** Automated ELISA system in place of semi automated ELISA system Western blot (preferably automated) Deep Freeze -70°C Equipment and reagents for PCR (as mentioned in Chapter 8).	**Note:** some of the specialised laboratories/ centres may need to undertake culture facilities for HIV. The necessary infrastructures are elaborate for HIV culture and are beyond the scope of discussion in this manual.

***Note:** At present there is hardly any documented guideline regarding the requirement of space for HIV testing laboratories in India. Thus, based on the consultations among the experts an attempt has been made to provide some idea in this regard. However, some broad components regarding specification of rooms, for optimal performance of the laboratory are mentioned below:

Broad specifications of the rooms:-

- Rooms should be adequately ventilated and well lighted.
- Floors should be impervious and easy to clean.
- There should be no open drainage system.
- Rooms should be made rodent free and insect free.
- All rooms should be either centrally air-conditioned or fitted with window air-conditioners.
- Rooms for guest, for eating etc should be away from the laboratory area.
- Adequate number of electricity points should be provided so that any equipments purchased later on can be used without additional electrical works.

Chapter 17

POST EXPOSURE PROPHYLAXIS GUIDELINES

17.1 INTRODUCTION

Health care workers (HCW) are normally at a very low risk of acquiring HIV infection during management of the infected patient. However, inspite of a low statistical risk of acquisition of HIV, the absence of a vaccine or effective-curative treatment, makes the health care worker apprehensive. So, it is very necessary to have a comprehensive programme in place to deal with anticipated accidental exposure.

Most exposures do not result in infection. The risk of infection varies with type of exposure and other factors such as:

- The amount of blood involved in the exposure
- The amount of virus in patient's blood at the time of exposure
- Whether post exposure prophylaxis (PEP) was taken within the recommended time.

Prevention is the mainstay of strategy to avoid occupational exposure to blood/body fluids. All the biosafety precautions emphasized in chapter 4 must be practised at all times for all patients, blood and body fluids while providing medical services.

17.2 DEFINITION OF AN OCCUPATIONAL EXPOSURE

An occupational exposure that may place a worker at risk of HIV infection is a percutaneous injury, contact of mucous membrane or contact of skin (especially when the skin is chapped, abraded or afflicted with dermatitis or the contact is prolonged or involving an extensive area) with blood, tissue or other body fluids to which universal precautions apply.

17.3 STEPS TO BE TAKEN ON EXPOSURE TO HIV INFECTED BLOOD/BODY FLUIDS AND CONTAMINATED SHARPS ETC.

Immediately following an exposure:

- Needlesticks and cuts should be washed with soap and water;
- Splashes to the nose, mouth or skin, should be flushed with water;
- Eyes should be irrigated with clean water; saline, or sterile irrigants;
- Pricked finger should not be put into mouth, reflexly.

No scientific evidence exists as to the fact that the use of antiseptics for wound care or squeezing the wound will reduce the risk of transmission of HIV. However, this must always be done. The use of a caustic agent such as bleach is not recommended (Annexure 17).

Report the exposure to the appropriate authority and condition must be treated as an emergency. Prompt reporting is essential because in some cases, HIV postexposure

prophylaxis (PEP) may be recommended and it should be started as soon as possible, preferably within a few hours.

Based on animal models, the success of PEP therapy is reported to be maximal when started within a matter of hours after the exposure. Although, any cutoff time is arbitrary initiating treatment more than 72 hours after the exposure is not recommended. Although perhaps not as effective as prophylaxis, late PEP (after 72 hours) may still be useful as early treatment of HIV infection, in case infection has occured.

17.4 TYPES OF OCCUPATIONAL EXPOSURES TO HIV FOR WHICH PEP IS RECOMMENDED

Most occupational exposures do not lead to HIV infection. The chance of possible serious side effects (toxicity) of the drugs used to prevent infection may be much greater than the chance of HIV infection from some kinds of exposures. Both risk of infection and possible side effects of drugs should be carefully considered when deciding whether to take postexposure prophylaxis. Exposures with a lower infection risk may not be worth the risk of the side effects associated with these drugs. The decision to start PEP is made on the basis of degree of exposure to HIV and HIV status of the source from whom exposure/ infection has occurred: (**Fig. 17.1, 17.2, 17.3**).

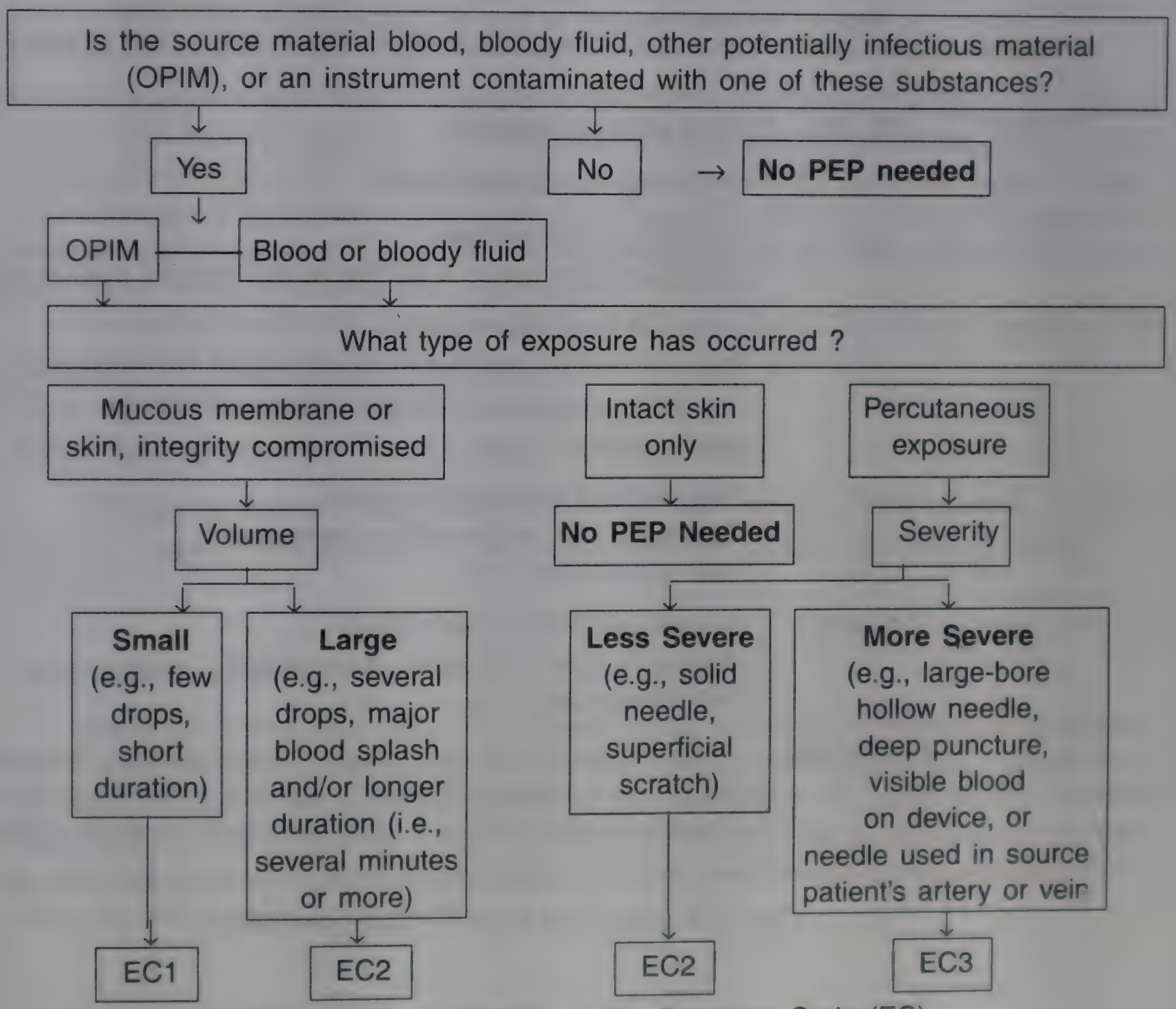

Is the source material blood, bloody fluid, other potentially infectious material (OPIM), or an instrument contaminated with one of these substances?
Yes
No
No PEP needed
OPIM
Blood or bloody fluid
What type of exposure has occurred ?
Mucous membrane or skin, integrity compromised
Intact skin only
Percutaneous exposure
Volume
No PEP Needed
Severity
Small (e.g., few drops, short duration)
Large (e.g., several drops, major blood splash and/or longer duration (i.e., several minutes or more)
Less Severe (e.g., solid needle, superficial scratch)
More Severe (e.g., large-bore hollow needle, deep puncture, visible blood on device, or needle used in source patient's artery or vein
EC1
EC2
EC2
EC3

Fig. 17.1 Determination of the Exposure Code (EC)

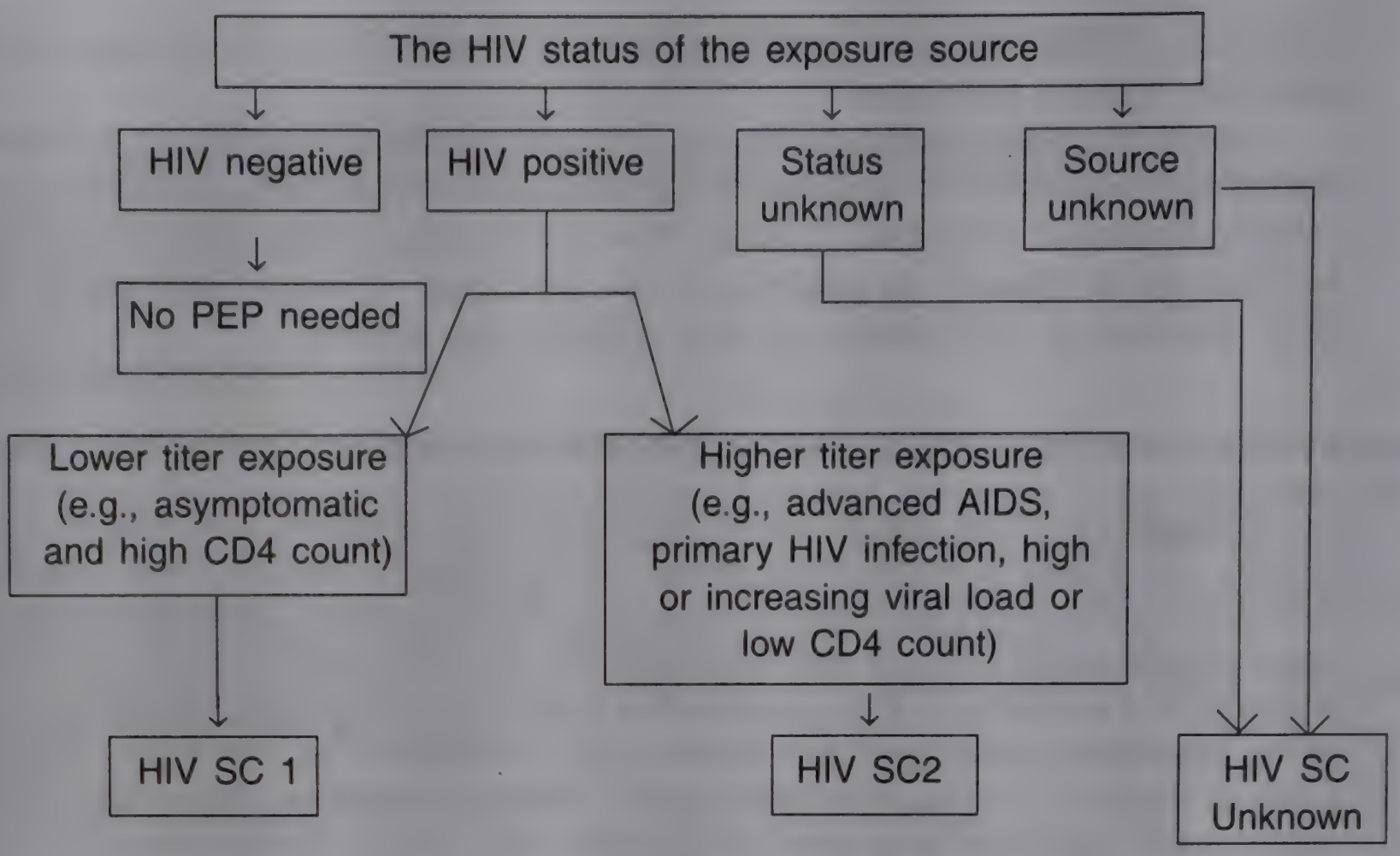

The HIV status of the exposure source
HIV negative
HIV positive
Status unknown
Source unknown
No PEP needed
Lower titer exposure (e.g., asymptomatic and high CD4 count)
Higher titer exposure (e.g., advanced AIDS, primary HIV infection, high or increasing viral load or low CD4 count)
HIV SC 1
HIV SC2
HIV SC Unknown

Fig. 17.2 Determination of the HIV Status Code (HIV SC)

EC	HIV SC	PEP recommendation
1	1	PEP may not be warranted
1	2	Consider basic regimen. Exposure type poses a negligible risk for HIV transmission.
2	1	Recommend basic regimen Most HIV exposures are in this category ; no increased risk for HIV transmission has been observed but use of PEP is appropriate.
2	2	Recommend expanded regimen. Exposure type represents an increased HIV transmission risk.
3	1 or 2	Recommend expanded regimen. Exposure type represents an increased HIV transmission risk.
	UNKNOWN	If the source, (in the case of an unknown source), the setting where the exposure occurred suggests a possible risk for HIV exposure and the EC is 2 or 3, consider PEP basic regimen.

Fig. 17.3 Determine the PEP recommendation

17.5 PRE AND POST TEST COUNSELLING AND TESTING

The person should be provided with pre test counselling and AZT be started as discussed above. Before starting AZT, 3-5 ml of person's reference blood sample is taken and tested for anti-HIV antibodies immediately after the exposure. In case the sample tests positived as per the strategy of HIV testing the individual is referred to the clinician for management, as a case of HIV infection. In case the sample tests non reactive, a 2nd sample is collected at 6 weeks and 3rd at 12 weeks after the exposure and tested for HIV-antibodies. The facilities for RT-PCR are available presently at MC Medical College, Mumbai and NARI, Pune and AIIMS, New Delhi and this can give us results even at 2nd or 4th weeks after exposure. Post test counselling is done in all cases.

During the follow-up period, especially the first 6-12 weeks when most infected persons are expected to show signs of infection, the recommendations for preventing transmission of HIV are to be followed by the HCW. These include refraining from blood, semen, organ donation and abstaining from sexual intercourse. In case sexual intercourse is undertaken a latex condom must be used correctly and consistently. This reduces the risk of HIV transmission. In addition, women should not breast-feed their infants during the follow-up period after exposure to prevent exposing their infants to HIV in breast milk.

17.6 DRUGS RECOMMENDED FOR POSTEXPOSURE PROPHYLAXIS/TREATMENT

It is recommended that in India zidovudine (ZDV) and lamivudine (3TC) be used as follows. Both these drugs should be considered for treatment of all exposures involving HIV-infected blood, fluid containing visible blood, or other potentially infectious fluids or tissues (ZDV-200 mg x 8 hly). Used in combination, ZDV and 3TC are very effective in treating HIV infection after exposure and considerable information shows, that they are safe when used for a short time (Lamivudine 150 mg x 12 hrly).

In selected cases (HIV status code to and EC code 2 and 3), Indinavir (or one of the other protease inhibitors) is also being used as per PEP guideline.

17. 7 DURATION FOR WHICH DRUGS NEED TO BE TAKEN

The optimal course of treatment is unknown; since 4 weeks of ZDV appears to provide protection against HIV infection, if tolerated, treatment should probably be taken for 4 weeks.

17.8 PREGNANCY AND PEP

Based on limited information, ZDV taken during 2nd or 3rd trimesters of pregnancy has not caused serious side effects in mothers or infants. There is very little information on the safety of ZDV when taken during the 1st trimester or on the safety of other antiviral drugs taken during pregnancy. If the HCW is pregnant at the time of occupational exposure to HIV, the designated authority/physician must be consulted about the use of anti-viral drugs for post exposure treatment.

17.9 FACTS KNOWN ABOUT THE SAFETY AND SIDE EFFECTS OF THESE DRUGS

Most of the information known about the safety and side effects of these drugs is based on studies of their use in HIV-infected individuals. For these individuals, ZDV and 3TC have usually been tolerated well except for nausea, vomiting, diarrhoea, tiredness, or headache for people taking ZDV.

17.10 STEPS TO BE UNDERTAKEN BY THE INFECTION CONTROL OFFICER ON RECEIVING INFORMATION ABOUT OCCUPATIONAL EXPOSURE

- All the needle stick injuries should be reported to the AIDS State Society giving the exposure code and the HIV status code in a proforma enclosed (Annexure 17.1).
- The State AIDS Societies should in turn inform NACO about the cases periodically.
- A registry is plannned to be opened in NACO soon, for follow-up of all such cases.
- NACO has decided in principle to supply antiretroviral drugs to all cases for post exposure prophylaxis in Govt. hospital settings for HCW.
- Infection control officers in all hospital have been directed to ensure that anti retroviral drugs for PEP are available in casualty at all the times.

Chapter 17 – Annexure 1

Management of sharps accidents

Each health facility should develop standard operating procedures to be followed by all health personnel in the case of sharps injury or other exposure.

In case of injury with a used needle or other sharp or if blood/body fluid is splashed into the mouth, eyes or onto broken skin, carry out the following procedure.

1. Needlepricks, cuts, bites or scratches.

(a) Encourage bleeding by squeezing.

(b) Wash thoroughly with soap and water.

c) Cover with a waterproof dressing.

2. Splashes to mouth or eyes.
Rinse thoroughly with plenty of runningwater

Proforma for reporting exposure to blood/body fluids.

Needle Stick Sharp Injury Protocol

Name of H.C.W.	:	
Section of HCW	:	
Employment No.	:	
Date of Needle Stick/Sharp Injury	:	
Date of Reporting to Casualty	:	
Site & Depth Of Injury		
Nature of Injury	:	Needle Prick/Sharp Cut/Laceration/Splash of Fluids Splattered Glass
Action taken in casualty		
Hep. B. vaccination given		Yes/No
HBIG		Yes/No

History of injury

History of Hepatitis B Immunization	:	Date: Intradermal/Intramuscular
Anti HBs Titre (Date of recent testing)		
01. HBsAg		Yes/No
02. HIV		Yes/No

Source of Injury (If Available)

Serum sent for	:	(reports to be entered in full: upon visit)
01. HIV		
02. HBsAg		

TESTS WHICH HAVE BEEN EVALUATED BY WHO COLLABORATING CENTRE ON AIDS IN THE DEPARTMENT OF MICROBIOLOGY, INSTITUTE OF TROPICAL MEDICINE, ANTWERP, BELGIUM

The list of assays given below (ELISA/Rapid/Supplemental) is relevant for use in India as these assays detect antibodies to both HIV-1 as well HIV-2. The list has been prepared from WHO document number WHO/BLS/98.1 with due permission from WHO.

18.1 ELISA/EIA FOR THE DETECTION OF ANTIBODY TO HIV-1 AND HIV-2

Manufacturer	Sensitivity	Specificity	d values		Reader/ visual (R/V)	Suitability
			WB+ sera	WB-sera		
Detect-HIV™ (Biochem Immunosystems)	100.0 (98.6-100.0)	97.4 (94.0-99.2)	12.65	-2.21	R	LS
DuPont HIV-1/HIV-2 ELISA (DuPont de Nemours)	100.0 (98.7-100.0)	85.6 (79.8-90.2)	9.34	-0.96	R	LS
Anti-HIV-1/HIV-2 EIA <Roche> (F. Hoffman-LaRoche)	100.0 (98.7-100.0)	96.9 (93.4-98.9)	11.30	-2.37	R	LS
Wellcozyme HIV-1+HIV-2 (Wellcome Diagnostics)	100.0 (98.7-100.0)	96.9 (93.3-98.9)	38.51	-1.99	R	LS
Peptide HIV ELISA (Cal-Tech Diagnostics)	72.6 (69.4-77.6)	95.4 (91.3-97.9)			V	S
Genelavia Mixt (Sanofi Diagnostics Pasteur)	100.0 (98.6-100.0)	98.5 (95.6-99.8)	16.77	-2.10	R	LS
Biotest Anti-HIV-1/-2 Recombinant (Biotest)	100.0 (98.6-100.0)	97.9 (94.9-97.4)	50.47	-3.08	R	LS
Enzymum-Test Anti-HIV-1+2 (Boehringer Mannheim)	100.0 (98.7-100.0)	100.0 (98.6-100.0)	5.50	-2.48	R	S
Innotest HIV-1/HIV-2 Ab (Innogenetics)	100.0 (98.8-100.0)	97.9 (95.9-99.9)	7.22	-2.30	R	LS
Clonatec HIV (1+2) Ab EIA (Clonatec)	99.6 (98.8-100.0)	95.9 (93.1-98.7)	7.47	-1.68	R	S

Peptide HIV-1 & HIV-2 ELISA Test (Sero-Immuno Dianostics)	97.6 (95.7-100.0)	98.5 (96.7-100.0)			V	LS
Cobas Core Anti-HIV-1/ HIV-2 EIA <Roche> (Hoffmann-La Roche)	100.0 (98.6-100.0)	89.2 (84.6-93.8)	10.8	-1.0	R	LS
Abbott Recombinant HIV-1/HIV-2 3rd Generation (Abbott)	100.0 (98.5-100.0)	100.0 (98.5-100.0)	11.5	-4.3	R	LS
HIV-1 and/or HIV-2 Recombige EIA (Cambridge Diagnostics)	100.0 (98.6-100.0)	100.0 (98.6-100.0)	10.4	-50	R	LS
UBI HIV-1/2 EIA 2nd (United Biomedical)	99.5 (98.6-100.0)	92.4 (88.6-96.2)	4.8	-1.5	R	S
HIV1+2 env Peptide EIA (Labsystem OY)	100.0 (98.6-100.0)	76.2 (70.0-82.4)			R	LS
VIDAS HIV-1+2 (Bio Merieux)	100.0 (98.5-100.0)	97.8 (95.6.100.0)			R	S
VIRONOSTIKA HIV Uni-Form II (Organon Taknika)	100.0 (99.6-100.0)	98.8 (97.6-100.0)	7.4	-3.0	R	LS
BIOTEST Anti-HIV-1/2 recombinant (Biotest AG)	100.0 (99.6-100.0)	99.1 (98.1-100.0)	74.9	-3.3	R	LS
INNOTEST HIV-1/HIV-2 Ab s.p. (Innogenetics n.v.)	100.0 (99.6-100.0)	98.8 (97.6-100.0)	14.0	-3.8	R	LS
UBI HIV 1/2 EIA (United Biomedical Inc.)	(99.6-100.0) 100.0	(99.7-100.0) 100.0	10.8	-3.2	R	LS
HIVisual 1 & 2 (Immuno Diagnostic Inc.)	90.9 (87.4-94-4)	94.9 (92.5-97.3)	1.88	-1.15	R	LS
HIV-1/HIV-2 ELISA (Genelabs Diagnostics)	100.0 (99.6-100.0)	97.3 (95.6-99.0)	72.2	12.7	R	LS
ETI-AB-HIV-1/2K (Sorin Biomedica)	100.0 (99.6-100.0)	98.8 (97.6-100.0)	10.39	-2.51	R	LS
HIV SCREEN (Labsystems OY)	100.0 (99.6-100.0)	99.7 (99.1-100.0)	21.54	-4.11	R	LS
HIV EIA (Labsystems OY)	100.0 (99.6-100.0)	99.4 (98.6-100.0)	14.20	-3.85	R	LS

18.2 SIMPLE/RAPID ASSAYS FOR THE DETECTION OF ANTIBODY TO HIV-1 AND HIV-2

Manufacturer	Sensitivity	Specificity	Ease of performance	Suitability
Test Pack HIV-1/HIV-2 Ab (Abbott)	100.0 (98.5-100.0)	95.9 (92.0-98.2)	VE	VS
HIV CHECK1+2/HIVSPOT 1+2 (DuPont de Nemours) (Genelabs Diagnostics)	99.3 (97.4-99.9)	100.0 (98.1-100.0)	E	VS
Clonatec rapid HIV-1-HIV-2 Ab (Clonatec)	98.9 (96.8-99.8)	99.5 (97.2-99.8)	E	VS
Recombigen HIV-1/HIV-2 Rapid Test Device (Cambridge Diagnostics)	100.0 (98.7-100.0)	94.5 (91.2-97.8)	E	VS
Serodia - HIV-1/2 (Fujirebio)	100.0 (98.5-100.0)	100.0 (98.5-100.0)	LE	S
SPAN COMBAIDS VISUAL (Span Diagnostics)	96.5 (93.5-99.5)	100.0 (98.3-100.0)	E	VS
CAPILLUS HIV-1/HIV-2 (Cambridge Diagnostics)	100.0 (99.6-100.0)	98.8 (97.6-100.0)	VE	VS
Immunocomb II BiSpot HIV1 & 2 (PBS Orgenics)	100.0 (99.6-100.0)	99.7 (99.1-100.0)	VE	VS
MicroRed HIV-1/HIV-2 Ab Test (Agen Biomedical)	98.5 (97.0-100.0)	95.5 (93.2-97.7)	VE	S
SimpliRed HIV-1/HIV-2 Ab Test (Agen Biomedical)	99.2 (98.2-100.0)	87.3 (83.7-90.9)	VE	S
HIV (Sav) 1 & 2 Rapid Sero Test (Diatech Diagnostica Ltd./ Healthcare Technologies Ltd.)	97.7 (95.9-99.5)	96.7 (94.8-98.6)	VE	S
ENTEBE HIV Dipsticl (Hepatika Laboratories)	100.0 (99.6-100.0)	96.4 (94.4-98.4)	E	S
Dipstick-HIV 1+2 (Immuno-Chemical Laboratories)	100.0 (99.6-100.0)	98.2 (96.8-99.6)	E	VS
SPAN COMBAIDS VISUAL (Span Diagnostics Ltd.)	100.0 (99.6-100.0)	88.0 (84.5-91.5)	E	S
DIA (Dot Immuno Assay) HIV-1 + HIV-2 (Weiner Lab.)	99.6 (98.8-100.0)	99.4 (98.6-100.0)	E	VS

18.3 SUPPLEMENTAL ASSAYS FOR THE DETECTION OF ANTIBODY TO HIV-1 AND HIV-2

Manufacturer	Sensitivity	Specificity	Ease of performance	Suitability
INNO-LIA HIV-1/HIV-2 Ab (Innogenetics)	100.0 (98.6-100.0)	100.0 (98.0-100.0)	LE	S
HIV CHECK1+2/HIVSPOT 1+2 (DuPont de Nemours) (Genelabs Diagnostics)	100.0 (99.4-100.0)	66.4 (57.9-74.1)	LE	S
Clonatec rapid HIV-1-HIV-2 Ab (Clonatec)	99.3 (96.4-99.9)	100.0 (98.1/100.0)	LE	S

VE = Very easy
LE = Less easy
LS = Less suitable
S = Suitable
VS = Very suitable

Chapter 19

SUGGESTED READINGS

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2. HIV-Volume 1: A Practical Approach; Virology and Immunology. Edited by J. Karn. Oxford University Press. Oxford 1995.
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4. Immunology of HIV Infection. Edited by Sudhir Gupta. Plenum Publishing Corporation, New York 1996.
5. AIDS/HIV: Reference Guide for Medical Professionals. Editors John L. Fahey and Diana Shin Flemming. Williams and Wilkins. A waverly company, Baltimore, 1996.
6. Guidelines for Preventing HIV,HBV and other Infections in the Health Care Setting. Document No. ISBN 974-558-7M-5. 1996.
7. Operational Characteristics of Commercially available Assays to Determine Antibodies to HIV-1 and/or HIV-2 in Human Sera. Document No. WHO/BLS/98.1 UNAIDS/98.14.
8. Revised Recommendations for the Selection and use of HIV antibody tests. WHO/UNAIDS, 1998.
9. National HIV Testing Policy. NACO Document, 1995.
10. Retroviral Testing: Essentials For Quality Control and Laboratory Diagnosis. Editors Neil T. Constantine. Johnny D. Callahan and Donglas M Watt. CRC Press. Ann Arbor, 1992.
11. Human Immunodeficiency Virus: Laboratory Training Manual. ASTPHLD, Connecticut Ave. Washington D.C. 1994.
12. Public health service guidelines for management of health care worker exposure to HIV and recommendations for post exposure prophylaxis MMWR, Vol. 47, NORR-7, May 15, 1998.
13. Kartz MH and Gerberding JL. Post exposure treatment of people exposed to the HIV through sexual contact or infection during sex. NEJM. 336 (15): 1097-99,1997.
14. National HIV testing network operational guidelines NACO Document.

Chapter 20

PROFORMAS TO BE USED FOR SENDING REPORTS TO DIRECTOR, STATE AIDS SOCIETY AND ADDITIONAL DIRECTOR TECHNICAL, NATIONAL AIDS CONTROL ORGANISATION

Monthly laboratory report on serological testing
Voluntary blood testing/surveillance centres

Name of the Testing Centre/Laboratory _____

City _____ State _____

Date and year of report _____ Report for the month of/yr. _____

Population/Area	Number of persons tested					
	By one test			By supplemental tests		
	Tested	Positive	Per cent	Tested	Positive	Per cent
STD clinic attendants						
Males						
Females						
Commercial sex workers						
Males						
Females						
Homosexuals						
Injecting drug users						
Males						
Females						
Mobile men, specify						
Antenatal, clinic attendants						
Blood donors, all						
Voluntary						
Replacement						
Suspect AIDS/ARC cases						
Other groups, specify:						
Not specified,						
Total, during this month						
Total, since inception						
Total, during						
For reference lab. only						
No. of confirmatory/supplementary tests						
No. of tests for quality control						

To be sent to Addl. Director Technical, NACO, G.O.I. during the 2nd wk. of every month.

Monthly laboratory report on serological testing Reference Centre

Name of the Testing Centre _____

City _____ State _____

Date and year of report _____ Report for the month of/yr. _____

Age/sex distribution of HIV tested persons

Age group	Tested by one screening test		Tested by supplemental tests			
	By one test		Females		Both sexes	
	Tested	Positive	Tested	Positive	Tested	Positive
0-10						
11-20						
21-30						
31-40						
41 and above						
All ages						

Test kits supply

Test kit type and number of tests per kit	Received this year	Used by report date	In stock	Replacement required

Comments, problems _____

_____

Reporting Officer _____
(Name & Designation)

Date: _____ Signature: _____

To be sent to Addl. Director, Technical, NACO, MH & FW, Nirman Bhavan, New Delhi-110011 during the first weeek of every month.

Monthly state report on HIV serological testing
Voluntary blood testing/surveillance

Name of the Testing Centre/Laboratory _____

City _____ State _____

Date and year of report _____ Report for the month of/yr. _____

Population/Area	Number of persons tested					
	By one test			By supplemental tests		
	Tested	Positive	Per cent Positive	Tested	Positive	Per cent Positive
STD clinic attendants						
Males						
Females						
Commercial sex workers						
Males						
Females						
Homosexuals						
Injecting drug users						
Males						
Females						
Mobile men, specify						
Antenatal, clinic attendants						
Blood donors, all						
Voluntary						
Replacement						
Suspect AIDS/ARC cases						
Other groups, specify:						
Not specified,						
Total, during this month						
Total, since inception						
Total, during the year						

For reference centres

No. of confirmatory/supplementary tests						
No. of tests for quality control						

To be sent to State AIDS Programme Officer by each testing facility during the first wk. of every month.

Monthly donors screening report of blood bank/transfusion centre

Blood bank/transfusion centre _____

City _____ State _____ Date of report/yr. _____

Report for the month of _____ year _____

Name of the centre/ No. of tests performed/ No. of positive	No. of samples from donors								
	Voluntary			Replacement			Total		
	M	F	T	M	F	T	M	F	T
ZBTC									
No. Tested									
No. Positive									
Blood banks attached to ZBTC									
1.									
No. Tested									
No. Positive									
2.									
No. Tested									
No. Positive									
3.									
No. Tested									
No. Positive									
4.									
Tested									
Positive									
5.									
Tested									
Positive									
6.									
Tested									
Positive									
Total for the reporting month									
Total since inception									
Total during the current year									

TOTAL DURING THE PREVIOUS YEAR (.....)

Monthly donors screening report of blood bank/transfusion centre

Blood bank/transfusion centre _____

City _____ State _____ Date and year of report _____

Report for the month of _____ Year _____

HIV testing protocol: One screening test (E/R/S) _____ two or more (E/R/S)_____

Three (E/R/S) _____ (E/R/S) and Western Blot tests

Tested and results		No. of samples from donors								
		Voluntary			Replacement			Total		
		M	F	T	M	F	T	M	F	T
HBs Ag:	Tested									
	Positive									
VDRL:	Tested									
	Positive									
MALARIA:	Tested									
	Positive									

Tests performed during the calendar year

Tests	Voluntary/Replacement		
	M	F	T
HBs Ag:			
VDRL			
Malaria			

HIV test kits supply

Test kit type and No. of Tests per kit	Received this year	Used by reporting time	In stock	Replacement required

Problems, comments _____

Reporting Officer _____
(Name & Designation)

Date: _____ Signature _____

Monthly donors screening report of blood bank/transfusion centre

State _____

Report for the month of _____ year _____

Name of the centre/ No. of tests performed/ No. of positive	No. of samples from donors											
	Voluntary			Replacement			Professional			Total		
	M	F	T	M	F	T	M	F	T	M	F	T
ZBTC												
Tested												
Positive												
Blood banks attached to zbtc												
1.												
Tested												
Positive												
2.												
Tested												
Positive												
3.												
Tested												
Positive												
4.												
Tested												
Positive												
5.												
Tested												
Positive												
6.												
Tested												
Positive												
Total for reporting month												
Total since inception												
Total the year												

TOTAL DURING